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(54) Title: HYDROGEL COMPOSITIONS FOR USE IN CELL EXPANSION AND DIFFERENTIATION

(57) Abstract: Hydrogel compositions and methods of using hydrogel compositions are disclosed. Advantageously, the hydrogel compositions offer the ability to promote cellular expansion and/or cellular differentiation of various cells.

HYDROGEL COMPOSITIONS FOR USE IN CELL EXPANSION AND DIFFERENTIATION

Statement in Support for Filing a Sequence Listing

A paper copy of the Sequence Listing and a computer readable form of the 5 Sequence Listing containing the file named "P150263US01_ST25.txt", which is 11,615 bytes in size (as measured in MICROSOFT WINDOWS® EXPLORER), are provided herein and are herein incorporated by reference. This Sequence Listing consists of SEQ ID NOs:1-48.

Cross-Reference to Related Applications

This application claims priority to U.S. Provisional Patent Application No. 10 61/978,032, filed on April 10, 2014, which is incorporated by reference herein in its entirety.

Statement Regarding Federally Sponsored Research or Development

This invention was made with government support under HL093282 awarded by the National Institutes of Health. The government has certain rights in the invention.

Background of the Disclosure

15 The present disclosure relates generally to methods for preparing biomaterial compositions and methods for using the biomaterial compositions. More particularly, the present disclosure relates to hydrogel compositions and to methods for using the hydrogel compositions to promote cell expansion and cell differentiation.

20 The development of most tissue types involves a complex interplay of multiple signals leading to controlled precursor cell differentiation into mature, tissue-specific cell types. For example, mesenchymal stem cells (MSCs) may be differentiated *in vitro* into osteoblasts, chondrocytes, myoblasts, adipocytes, neurons, and endothelial cells by exposure to a variety of growth factors. Routine cellular expansion and differentiation protocols rely on high concentrations of expensive recombinant growth factors. Substantial 25 progress has been made in the development of defined media, but only more recently has the role of substrates and cell-substrate adhesion on cell growth been examined.

30 Currently, tissue culture polystyrene (TCPS) is the "gold standard" for cellular expansion and differentiation during *in vitro* cell culture, particularly, for human mesenchymal stem cells (hMSCs); however, TCPS does not allow the user to control substrate stiffness and growth factor regulation. Stiffness has been demonstrated to be important in controlling cellular proliferation, lineage specification, commitment, and maturation.

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Accordingly, there exists a need for methods for preparing biomaterial compositions that will support survival and growth of cells in culture, and particularly, to provide specific molecules that promote cellular expansion, cellular differentiation and regulate cellular behavior. It would further be advantageous if the biomaterial compositions 5 allowed for control over both biomaterial substrate stiffness and growth factor signaling.

Brief Description of the Disclosure

The present disclosure relates generally to biomaterial compositions and methods for using the biomaterial compositions. More particularly, the present disclosure relates to hydrogel compositions and methods for promoting cellular expansion and cellular 10 differentiation using the hydrogel compositions.

In accordance with the present disclosure, methods for preparing hydrogel compositions to support survival and growth of cells in culture have been discovered. The hydrogel compositions of the present disclosure can also be used for two-dimensional (2D) and three-dimensional (3D) cell culture. The hydrogel compositions of the present disclosure 15 can further be used for two-dimensional and three-dimensional enrichment of biomolecules such as, for example, biomolecules to cell surfaces using soluble factor binders. The hydrogel compositions further offer design control over both hydrogel substrate stiffness and growth factor signaling, allow for attachment with phenotypes consistent with those offered by conventional MATRIGEL®, and growth factor regulation.

20 In one aspect, the present disclosure is directed to a method of promoting cellular expansion. The method includes preparing a hydrogel composition, wherein the hydrogel composition includes a polyethylene glycol functionalized with norbornene, a crosslinking peptide, and a cell adhesion peptide; contacting a cell with the hydrogel composition; and culturing the cell.

25 In another aspect, the present disclosure is directed to a method of promoting cellular differentiation. The method includes preparing a hydrogel composition, wherein the hydrogel composition includes a polyethylene glycol functionalized with norbornene, a crosslinking peptide, and a cell adhesion peptide; contacting a cell the hydrogel composition; and culturing the cell.

30 Brief Description of the Drawings

The disclosure will be better understood, and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the

following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

FIGS. 1A-1B are schematic illustrations of the steps for preparing a hydrogel array of the present disclosure.

5 FIG. 2A is a schematic illustration of the steps for patterning a metal-coated substrate used in the method for preparing a hydrogel array of the present disclosure.

FIG. 2B are end view drawings of the metal-coated substrate during the steps for patterning a metal-coated substrate shown in FIG. 2A.

10 FIG. 3 is a photograph of a hydrogel array with 64 individual hydrogel spots prepared using the methods of the present disclosure.

FIG. 4 is a graph illustrating the surface roughness of a hydrogel array as determined by atomic force microscopy.

FIG. 5 illustrates high magnification top-view images showing different shapes of individual hydrogel spots.

15 FIG. 6 is a side-on image showing individual hydrogel spots having different heights.

FIG. 7 is a hydrogel array showing differential patterning of individual hydrogel spots by increasing the density of a fluorescently-tagged peptide and increasing the density of encapsulated fluorescent microspheres, as discussed in Example 2.

20 FIG. 8 is a graph illustrating control of the modulus of individual hydrogel spots of a hydrogel array by changing the total concentration of PEG-NB (w/w%) in the hydrogel precursor solution using the methods of the present disclosure.

FIG. 9 is a schematic illustrating the steps for preparing a hydrogel array and further assembling the hydrogel array with a microwell add-on using the methods of the 25 present disclosure.

FIG. 10A-10C are photographs of hMSCs cultured on hydrogel arrays prepared using 4 wt. % (FIG. 10A), 6 wt. % (FIG. 10B) and 8 wt. % (FIG. 10C) polyethylene glycol and presenting linear RGD peptide, as discussed in Example 2. Scale bar = 100 μ m.

30 FIG. 11A-11C are photographs of hESCs cultured on hydrogel arrays prepared using 4 wt. % (FIG. 11A), 6 wt. % (FIG. 11B) and 8 wt. % (FIG. 11C) polyethylene glycol and presenting varying peptide identity, as discussed in Example 2. Scale bar = 100 μ m.

FIGS. 12-14 depict hMSC cell attachment, spreading and proliferation as analyzed in Example 3.

FIGS. 15 & 16 depict hESC cell spreading and proliferation rates as analyzed in Example 4.

While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings 5 and are herein described below in detail. It should be understood, however, that the description of specific embodiments is not intended to limit the disclosure to cover all modifications, equivalents and alternatives falling within the scope of the disclosure as defined by the appended claims.

Detailed Description of the Disclosure

10 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the present disclosure, the preferred methods and materials are described below.

15 In accordance with the present disclosure, methods for preparing biomaterial compositions for promoting cellular expansion and differentiation have been discovered. More particularly, the present disclosure relates to hydrogel compositions. In one aspect, hydrogel compositions can be prepared as a hydrogel array with individually controlled hydrogel spot modulus, hydrogel spot polymer density, hydrogel spot ligand identity and 20 hydrogel spot ligand density and to methods for preparing the hydrogel arrays. In another aspect, the hydrogel compositions can be prepared as coatings such as for use on the surfaces of cell culture plates. In yet another aspect, the hydrogel compositions can be prepared as microcarriers in suspension culture. The hydrogel compositions of the present disclosure can be functionalized with biomolecules, are compatible with cell culture and are biocompatible. 25 The hydrogel compositions of the present disclosure can be used to alter (e.g., enhance, inhibit and change) cell function, and in particular, cellular expansion, maturation and differentiation.

30 As known by those skilled in the art, a hydrogel composition is a network of polymer chains that are hydrophilic in which a polymeric material and water are in an equilibrated form. The hydrogel composition is formed using unpolymerized starting components. The polymeric material can be, for example, a natural polymer material, a synthetic polymer material and combinations thereof.

The methods for preparing hydrogel compositions of the present disclosure advantageously allows for the direct incorporation of peptides into the hydrogel network during polymerization by including a cysteine in the amino acid sequence during synthesis, which allows for eliminating the need for post-synthetic modifications. In this way, peptides 5 can be utilized as crosslinkers by including cysteine on each end or they can be incorporated as pendant groups, which can be pre-coupled to the polymer backbone and mixed in varying combinations or incorporated during polymerization for simplicity.

Hydrogel Compositions and Methods for Preparing Hydrogel Compositions

The present disclosure is generally directed to methods for preparing a hydrogel composition and use of the resulting compositions. When used to prepare a 10 hydrogel array, the preparation methods generally include contacting a hydrogel precursor solution with a substrate, wherein the substrate includes a hydrophobic region and a hydrophilic region; placing a surface-modified substrate onto the hydrogel precursor solution such that the hydrogel precursor solution is located between the substrate and the surface-modified substrate; polymerizing the hydrogel precursor solution; and separating the surface-modified substrate from the substrate, to result in the hydrogel array. (See, FIGS. 1A-1B). 15 Thus, the polymer hydrogel precursor solution polymerizes between the substrate and the surface-modified substrate and the resultant hydrogel transfers with the surface-modified substrate such that the surface-modified substrate includes the hydrogel array. In one embodiment, the hydrogel array can be patterned to include an array of hydrogel spots 20 surrounded by a hydrogel-free background as described in more detail below. In another embodiment, the hydrogel array can be patterned such that an array of hydrogel-free spots (or pools) is formed within a hydrogel background as described in more detail below.

In hydrogel arrays having hydrogel spots, the resultant hydrogel array can be patterned to result in differential wettability to define the geometry of each hydrogel spot and 25 confine the contents of each hydrogel spot of the array, as well as define the spatial pattern of each hydrogel spot in the array in relation to neighboring spots. This is particularly useful for preparing hydrogel arrays for use with common microarray add-ons of different sizes and dimensions consistent with those of common multi-well plates (e.g., 96 well plates, 384 well plates, etc.) This is also useful for use with multichannel pipettes for enhanced-throughput 30 cell culture, media exchange, and the like. The individual hydrogel spots of the array can have any desired shape (see e.g., FIG. 5). For example, the shape can be circular, round, oval, quatrefoil, rectangular, triangular, star-shaped, diamond-shaped, combinations thereof,

and the like. Patterns of hydrogel spots may also be created in rows, spirals, circles, squares, rectangles, combinations thereof, and the like. The shape of the individual hydrogel spot can be varied by changing the pattern of the stencil used for etching during patterning of the patterned substrate.

5 In hydrogel arrays having hydrogel-free spots, the individual hydrogel-free spots can have any desired shape. For example, the shape can be circular, round, oval, quatrefoil, rectangular, triangular, star-shaped, diamond-shaped, combinations thereof, and the like. Patterns of hydrogel-free spots may also be created in rows, spirals, circles, squares, rectangles, combinations thereof, and the like. The shape of the individual hydrogel-free spot
10 can be varied by changing the pattern of the stencil used for etching during patterning of the patterned substrate.

15 The upper size limit of the hydrogel array depends on the dimensions of the patterned substrate and/or the dimensions of the surface-modified substrate. The resultant hydrogel array can also be patterned to result in individual hydrogel spots and hydrogel-free spots having any desired sizes. The size and shape of the individual hydrogel spot and hydrogel-free spot can be varied by changing the pattern of the stencil used for etching during patterning of the patterned substrate. Suitable individual hydrogel spot size of the hydrogel array can be small enough to accommodate a single cell, but also large enough to accommodate many cells, for example. Thus, the individual hydrogel spot size of the hydrogel array can have any desired diameter. Particularly suitable individual hydrogel spot sizes of the hydrogel array can be about 10 μm and larger.
20

25 A patterned substrate can be prepared by creating hydrophobic regions and hydrophilic regions formed by self-assembled monolayers (SAMs), such as described in US Patent Application Serial No. 14/339,938, filed on July 24, 2014, herein incorporated by reference to the extent it is consistent herewith. Suitable substrates for forming self-assembled monolayers are known to those skilled in the art and can be, for example, metal-coated substrates, silicon substrates, diamond substrates, polydimethylsiloxane (PDMS) substrates, and the like (as described in Love et al., *Chem. Rev.* 2005, 105:1103-1169, for example, which is hereby incorporated by reference to the extent its disclosure is consistent
30 with the present disclosure). The patterned substrate can be prepared, for example, by forming regions with differential wettability on a substrate by immersing the substrate in a perfluorinated alkanethiol solution to allow perfluorinated alkanethiolate self-assembled monolayers (fluoraSAMs) to form. To form hydrophilic regions, a stencil can be placed on

the fluoraSAMs metal-coated substrate to selectively protect regions of the fluoraSAMs metal-coated substrate from plasma etching. Exposed regions of the fluoraSAMs substrate can then be etched by oxygen plasma treatment to form etched fluoraSAMs in the substrate. The substrate is then immersed in a hydroxyl-terminated alkanethiol solution to form a 5 hydrophilic alkanethiolate SAM (EG₃SAM) in the etched regions of the substrate. The resulting patterned substrate possesses differential wettability based on the hydrophobic SAMs and hydrophilic SAMs.

The method can further include placing a spacer between the patterned substrate and the surface-modified substrate. The spacer placed onto the patterned substrate 10 while performing the method functions to define the height (or thickness) of the hydrogel forming the hydrogel array. A spacer may be particularly desirable when preparing higher (i.e., thicker) hydrogel arrays. Thus, the hydrogel array can have any desirable height (see e.g., FIG. 6). Suitable heights of the hydrogel array can be from about 20 micrometers (μm) to about 1 millimeter, however, hydrogel arrays can be made much higher than 1 millimeter 15 if desired. The spacer also functions to prevent direct contact between the surface of the patterned substrate and the surface-modified substrate during formation of the hydrogel. The spacer used in the method can be any suitable material known to those skilled in the art. A particularly suitable spacer can be, for example, polydimethylsiloxane (PDMS). The height 20 of the hydrogel array can be determined, for example, using a microscope to focus from the top of the hydrogel down to the substrate, using a microscope to focus from the substrate up to the top of the hydrogel, and by measuring the surface roughness of a hydrogel array as determined by atomic force microscopy (see e.g., FIG. 4).

The preparation method further includes contacting a hydrogel precursor 25 solution with the patterned substrate. In particular, the hydrogel precursor solution is contacted with the hydrophilic regions of the patterned substrate. The hydrophobic regions of the patterned substrate serve as a barrier between neighboring hydrophilic regions and also allow for the isolation of each hydrophilic region. The hydrogel precursor solution can be, for example, a combination of a polymer and a multifunctional polymer crosslinker.

When used as a hydrogel coating composition, preparation methods generally 30 include contacting a hydrogel precursor solution with a substrate to be coated (e.g., surface of a cell culture plate).

Suitable polymers for use in the hydrogel precursor solution are known by those skilled in the art and can include, for example, poly(ethylene glycol), hyaluronic acid,

gelatin, collagen, MATRIGEL®, dithiol polymers (e.g., acrylamide), click-based composite hydrogels (as discussed in Polizzotti et al. *Biomacromolecules* 2008, 9:1084-1087, which is hereby incorporated by reference to the extent its disclosure is consistent with the present disclosure), poly(ethylene glycol)-diacrylate, poly(ethylene glycol)-vinyl sulfone, and the like. Particularly suitable polymers can be, for example, poly(ethylene glycol). Particularly suitable polymers can be, for example, functionalized polymers. Functionalization of the polymer can be confirmed with ¹H nuclear magnetic resonance spectroscopy, mass spectroscopy, Elman's reagent, UV-Vis spectroscopy, infrared spectroscopy, and other methods known to those skilled in the art, for example.

A particularly suitable functionalized polymer can be, for example, eight-arm poly(ethylene glycol) with terminal hydroxyl (-OH) groups (commercially available from JenKem Technology USA, Allen, TX) that is functionalized with norbornene. Eight-arm poly(ethylene glycol) can be functionalized with norbornene as described in Fairbanks et al. (*Adv. Mater.* 2009, 21:5005-5010).

Other particularly suitable polymers are poly(ethylene glycols) that may be functionalized using click chemistry. "Click" chemistry is an extremely versatile method for chemically attaching biomolecules, which is used to describe the [3+2] cycloaddition between alkyne and azide functional groups. Azides and alkynes are largely inert towards biological molecules and aqueous environments, which allows the use of the Huisgen 1,3-dipolar cycloaddition to yield stable triazoles that are very difficult to oxidize or reduce. Both the copper(I)-catalyzed and copper-free strained-alkyne variant reactions are mild and very efficient. These reactions can also be performed in small volumes of aqueous solutions, are insensitive to oxygen and water, and robust to functional groups on peptides. Click chemistry allows for selectivity in conjugation reactions in biological samples such as, for example, oligonucleotides and proteins. Particularly suitable reagents for click chemistry are commercially available from Laysan Bio Inc. (Arab, AL).

Generally, the hydrogel precursor solutions include concentrations of polymer of up to, and including, 200 mg/mL, including from about 36 mg/mL to about 160 mg/mL, and including from about 36 mg/mL to about 70 mg/mL.

Suitable multifunctional polymer crosslinkers for use in the hydrogel precursor solution are known by those skilled in the art. In particular, the multifunctional crosslinker can be, for example, a bifunctional polymer crosslinker and a multifunctional polymer crosslinker ($n \geq 2$) and terminated with a functional group that can form a covalent

bond with the polymer of the hydrogel precursor solution. Particularly suitable bi-functional polymer crosslinkers and multifunctional polymer crosslinkers can be, for example, polyethylene glycol dithiol (PEG-DT), protease-degradable crosslinkers and multi-arm poly(ethylene glycol) terminated with thiol (e.g., 4-arm PEG terminated with thiol). Suitable 5 protease-degradable crosslinkers can be, for example, matrix metalloproteinase (MMP)-degradable crosslinkers as described in Nagase and Fields (Biopolymers 1996, 40:399-416, which is hereby incorporated by reference to the extent it is consistent with the present disclosure). More particularly, suitable MMP-degradable crosslinking peptides for use in the hydrogel precursor solution include KCGGPQGIWGQGCK (SEQ ID NO:27) and 10 KCGGPQGIAGQGCK (SEQ ID NO:28).

The hydrogel precursor solution can further include an initiator. As known by those skilled in the art hydrogel polymerization can occur in the absence of an initiator. An initiator can, however, induce polymerization and/or decrease the polymerization rate. Suitable initiators are known to those skilled in the art and can be, for example, chemical 15 initiators and photoinitiators. Particularly suitable photoinitiators can be, for example, IRGACURE 2959 photoinitiator (commercially available from Ciba/BASF, Ludwigshafen, Germany) and Eosin Y. Polymerization to form the hydrogel can also be performed by temperature change.

In another aspect, the hydrogel precursor solution can include a cell adhesion 20 peptide. As used herein, a “cell adhesion peptide” refers to an amino acid sequence obtained from an adhesion protein to which cells bind via a receptor-ligand interaction. Varying the cell adhesion peptide and concentrations thereof in the solution allow for the ability to control the stability of the cellular attachment to the resulting hydrogel composition. Suitable cell adhesion peptides include, for example, RGD, RGDS (SEQ ID NO:1), CRGDS (SEQ ID 25 NO:2), CRGDSP (SEQ ID NO:3), PHSRN (SEQ ID NO:4), GWGGRGDSP (SEQ ID NO:5), SIDQVEPYSSSTAQ (SEQ ID NO:6), GRNIAEIIKDI (SEQ ID NO:7), DITYVRLKF (SEQ ID NO:8), DITVTLNRL (SEQ ID NO:9), GRYVVLPR (SEQ ID NO:10), GNRWHSIYITRFG (SEQ ID NO:11), GASIKVAVSADR (SEQ ID NO:12), GTTVKYIFR (SEQ ID NO:13), GSIKIRGTYS (SEQ ID NO:14), GSINNNR (SEQ ID NO:15), 30 SDPGYIGSR (SEQ ID NO:16), YIGSR (SEQ ID NO:17), GTPGPQGIAGQGVV (SEQ ID NO:18), GTPGPQGIAGQRVV (SEQ ID NO:19), MNYYNS (SEQ ID NO:20), KKQFRHRNRKG (SEQ ID NO:21), CRGDGGGGGGGGGGPHSRN (SEQ ID NO:29), CPHSRNSGSGSGSGRGD (SEQ ID NO:30), Acetylated-GCYGRGDSPG (SEQ

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10 ID NO:31), CRDGS (SEQ ID NO:32), cyclic RGD{Fd}C (SEQ ID NO:33), RKRLQVQLSIRT (SEQ ID NO:37), IKAV (SEQ ID NO:38), YIGSR (SEQ ID NO:39), KRTGQQYKL (SEQ ID NO:40), TYRSRKY (SEQ ID NO:41), KRTGQQYKLGSKTGPGQK (SEQ ID NO:42), QAKHKQRKRLKSSC (SEQ ID NO:43), SPKHHSQRARKKKKNKNC (SEQ ID NO:44), XBBXBX, wherein B = basic residue and X = hydrophobic residue (SEQ ID NO:45), XBBBXXBX, wherein B = basic residue and X = hydrophobic residue (SEQ ID NO:46), and RGDSP (SEQ ID NO:47).

15 The concentration of cell adhesion peptide in the hydrogel precursor solution will depend on the specific cell adhesion peptide being used as well as the other components in the hydrogel precursor solution. Typically, however, the hydrogel precursor solution includes from about 0.125 mM to about 4 mM cell adhesion peptide, including from about 0.25 mM to about 2 mM cell adhesion peptide. In one suitable embodiment, the cell adhesion peptide is CRGDS (SEQ ID NO:2), and the hydrogel precursor solution includes from about 0.25 mM to about 4 mM CRGDS (SEQ ID NO:2). In another suitable 20 embodiment, the cell adhesion peptide is a cyclic RGD, and the hydrogel precursor solution includes from about 0.125 mM to about 2 mM cyclic RGD, particularly cyclic RGD{Fd}C (SEQ ID NO:33).

25 In another aspect, the hydrogel precursor solution can include a soluble factor binder. In one aspect, a peptide for binding a soluble factor contained in a cell culture medium is included in the hydrogel precursor solution. The density (concentration) of the soluble factor binder in a hydrogel composition can be controlled by altering the concentration of the soluble factor binder in the hydrogel precursor solution. Examples of particularly suitable soluble factor binders are provided in Table 1, below.

Table 1. Soluble factor binder peptide sequences for hydrogel compositions.

Name/Source	Sequence	SEQ ID NO:
Vascular Endothelial Growth Factor-Receptor Binding Peptide	GGGKLTWQELYQLKYKGI	22
Vascular endothelial growth factor receptor binding peptide (VR-BP)	KLTWQELYQLKYKGI	23
Bone morphogenetic protein-2 (BMP-2) receptor binding peptide	KIPKASSVPTEL	24
Bone morphogenic protein receptor-binding peptide	KIPKASSVPTELSAISTLYL	25
Heparin proteoglycan-binding peptide (HPG-BP)	KRTGQYKL	26
MMP-degradable peptide crosslinker	KCGGPQGIWGQGCK	27
MMP-degradable peptide crosslinker 2	KCGGPQGIAGQGCK	28
VEGF binding peptide	CE{Fd}{Ad}{Yd}{Ld}IDFNWEYPASK	35
Scrambled VEGF binding peptide	CD{Ad}PYN{Fd}EFAWE{Yd}VIS{Ld}K	36

The concentration of soluble factor binder in the hydrogel precursor solution
5 will depend on the specific soluble factor binder being used as well as the other components in the hydrogel precursor solution.

In another aspect, the hydrogel precursor solution can further include a cell. Suitable cells are known to those skilled in the art and can include, for example, an embryonic stem cell, an embryonic stem cell-derived neuron, an embryonic stem cell-derived neural progenitor cell, an embryonic stem cell-derived astrocyte, an embryonic stem cell-derived microglial cell, an embryonic stem cell-derived endothelial cell, an embryonic stem cell-derived retinal pigment epithelial cell, an induced pluripotent stem cell, an induced pluripotent stem cell-derived neural progenitor cell, an induced pluripotent stem cell-derived astrocyte, an induced pluripotent stem cell-derived microglial cell, an induced pluripotent stem cell-derived endothelial cell, a mesenchymal stem cell, an umbilical vein endothelial

cell, an NIH 3T3 fibroblast, a dermal fibroblast, a fibrosarcoma cell, a valvular interstitial cell, a cardiomyocyte, an induced pluripotent stem cell-derived cardiomyocyte, an endothelial progenitor cell, a circulating angiogenic cell, a neuron, a pericyte, a cancer cell, a hepatocyte, a pancreatic beta cell, a pancreatic islet cell and combinations thereof.

5 In another aspect, the hydrogel precursor solution can further include a microsphere carrier (i.e., microcarrier). Microsphere carriers can contain molecules such as, for example, cells, biomolecules, dyes and other molecules known to those skilled in the art. Microspheres can be degradable microspheres that dissolve or degrade to release the contents of the microsphere.

10 Once prepared, the hydrogel precursor solution is contacted with a substrate (e.g., a patterned surface-modified substrate, surface of a cell culture plate, etc.).

When used on a patterned surface-modified substrate, the surface-modified substrate can be, for example, mica, glass, silicon, diamond and metal oxide surfaces. The surface-modified substrate can be prepared, for example, by functionalizing a surface such as 15 a glass coverslip having a silane monolayer. A particularly suitable surface-modified substrate can be, for example, a glass slide. A particularly suitable method for functionalizing the substrate can be, for example, silanization. The substrate can be surface-modified by activating both sides of the surface in oxygen plasma treatment. Oxygen plasma treatment can increase the number of activated hydroxyl groups on the surface of the 20 substrate. As known by those skilled in the art, a silane monolayer can be prepared with an alkoxy silane that is dissolved in an anhydrous organic solvent such as, for example, toluene. Other suitable alkoxy silanes can be for example, aminosilanes, glycidoxy silanes and mercaptosilanes. Particularly suitable aminosilanes can be, for example, (3-aminopropyl)-triethoxysilane, (3-aminopropyl)-diethoxy-methylsilane, (3-aminopropyl)-dimethyl-25 ethoxysilane and (3-aminopropyl)-trimethoxysilane. Particularly suitable glycidoxy silanes can be, for example, (3-glycidoxypipyl)-dimethyl-ethoxysilane. Particularly suitable mercaptosilanes can be, for example, (3-mercaptopropyl)-trimethoxysilane and (3-mercaptopropyl)-methyl-dimethoxysilane. Other suitable silanes are commercially available (Sigma Aldrich, St. Louis, MO). Preparation of a surface-modified silane substrate can be 30 performed using any silane having a terminal functional group that can participate in click chemistry as described herein. For example, mercaptosilane contains a terminal thiol that can react with the norbornene of the PEG-norbornene. Other suitable functional surface-modified silane substrates can be, for example, acrylates and methacrylates. Following surface-

modification of the substrate, non-adhesive self-assembled monolayers are formed on the surface-modified substrate.

After contacting the substrate with the hydrogel precursor solution, the method includes polymerizing the hydrogel precursor solution such that polymerized hydrogel 5 attaches (i.e., is coupled) to the substrate.

In one embodiment, the method can be used to form an array having “spots” or “islands” of hydrogel (referred to herein as “hydrogel spots”) that are surrounded by a background that is substantially free, and even completely free, of hydrogel (“hydrogel-free”). In this embodiment, the hydrogel-free background corresponds to the hydrophobic 10 regions of the patterned substrate and the hydrogel spots correspond to the hydrophilic regions of the patterned substrate. Referring to FIG. 1, the circles would represent the hydrogel spots that would be surrounded by a hydrogel-free region in this embodiment.

In another embodiment, the method can be used to form an array having hydrogel-free pools surrounded by a background of hydrogel (referred to herein as “a hydrogel background”). Referring to FIG. 1, the circles would represent the hydrogel-free 15 pools that would be surrounded by the hydrogel-free background in this embodiment.

In another aspect, the present disclosure is directed to a hydrogel compositions including hydrogel spots having variable modulus, variable shear modulus, variable ligand identity, variable ligand density and combinations thereof. Hydrogel compositions having 20 variable modulus, variable shear modulus, variable ligand identity, variable ligand density and combinations thereof can be prepared according to the methods described herein above.

Suitable ligands are known to those skilled in the art and can be, for example, any biomolecule containing a cysteine and/or functionalized with a thiol. Thiol-functionalizing of ligands can be performed using commercially available kits (e.g., Traut’s 25 Reagent (2-iminothiolane•HCl), Thermo Fischer Scientific, Rockford, IL). Suitable ligands can be, for example, proteins, peptides, nucleic acids, polysaccharides, lipids, biomimetic materials and other molecules, and combinations thereof. Particularly suitable proteins can be, for example, adhesion proteins. Particularly suitable adhesion proteins can be, for example, fibronectin, cadherin and combinations thereof. Particularly suitable peptides can 30 be, for example, cell adhesion peptides and/or soluble factor binders, as described herein above.

Suitably, the hydrogel compositions of the present disclosure include combinations of cell adhesion peptides and soluble factor binders that are suspected of

binding or interacting with a cell to affect cell attachment, spreading, migration, maturation, proliferation, differentiation, and formation of cellular structures (e.g., tubules).

Hydrogel compositions may further include variable moduli. Hydrogel compositions can have a range of stiffness (expressed herein as substrate elastic moduli). For 5 example, hydrogels with different moduli can be prepared by changing the concentration of the polymer and/or changing the stoichiometric ratio of the multifunctional polymer (e.g., the bifunctional polymer thiol-polyethylene glycol-thiol (SH-PEG-SH)) to polymer ratio in the hydrogel precursor solution (see e.g., FIG. 8). Suitable ratios can be from about 1:1 to about 4:1 (molar ratio).

10 In another aspect, the patterned hydrogel array can be further assembled with a microarray add-on whereby the patterned hydrogel array is prepared with dimensions to accommodate add-ons of any size. Suitable microarray add-ons are commercially available (Grace Bio Labs, Bend, OR). A microarray add-on can allow for the isolation of each individual hydrogel spot and hydrogel-free pool of the hydrogel array such that soluble factor 15 presentation can be controlled. The microarray add-on can include the same number of openings as the number of individual hydrogel spots and hydrogel-free pools of the hydrogel array such that each hydrogel spot and hydrogel-free pool can be independently interrogated with soluble factor presentation. Alternatively, the microarray add-on can have larger openings that can accommodate more than one individual hydrogel spot and more than one 20 individual hydrogel-free pool. For example, a microarray add-on can have openings large enough to accommodate a single hydrogel spot or a single hydrogel-free pool.

Methods of Using the Hydrogel Compositions

25 In another aspect, the present disclosure is directed to methods of using the hydrogel compositions to promote cellular expansion, maturation and cellular differentiation. Generally, the methods include preparing the hydrogel compositions; contacting a cell with the hydrogel compositions; and culturing the cells. The hydrogel compositions are prepared as described above and typically include a polymer (e.g., a polyethylene glycol functionalized with norbornene), a multifunctional polymer crosslinker (e.g., MMP-degradable crosslinking peptide, non-degradable PEG-dithiol crosslinker), and a cell adhesion peptide as described 30 more fully above.

The method further includes contacting a cell with the hydrogel composition. As used herein, “contacting a cell” refers to seeding the cells with the purpose of culturing

the cells. As known by those skilled in the art a cell suspension is typically transferred to a substrate and cells are given sufficient time to adhere to the substrate.

In another embodiment, cells can be incorporated into the hydrogel of the hydrogel compositions using a hydrogel precursor solution that includes the polymer, the 5 crosslinker, the cell adhesion peptide, and the cell.

The cells are then cultured for a desired time such as, for example, about one hour to about 30 days. After the desired time, cells can be analyzed by microscopy such as, for example, immunofluorescence microscopy, phase contrast microscopy, light microscopy, electron microscopy and combinations thereof. Cells can be analyzed for cell attachment, 10 cell spreading, cell morphology, cell proliferation, cell migration, cell expansion, cell differentiation, protein expression, cell-to-cell contact formation, sprouting, tubulogenesis, formation of structures, and combinations thereof.

Suitable cells can be any cell known by those skilled in the art. Particularly suitable cells can include, for example, an embryonic stem cell, an embryonic stem cell-derived neuron, an embryonic stem cell-derived neural progenitor cell, an embryonic stem cell-derived astrocyte, an embryonic stem cell-derived microglial cell, an embryonic stem cell-derived endothelial cell, an embryonic stem cell-derived retinal pigment epithelial cell, an induced pluripotent stem cell, an induced pluripotent stem cell-derived neural progenitor cell, an induced pluripotent stem cell-derived astrocyte, an induced pluripotent stem cell-derived microglial cell, an induced pluripotent stem cell-derived endothelial cell, an induced pluripotent stem cell-derived retinal pigment epithelial cell, a mesenchymal stem cell, an umbilical vein endothelial cell, an NIH 3T3 fibroblast, a dermal fibroblast, a fibrosarcoma cell, a valvular interstitial cell, a cardiomyocyte, an induced pluripotent stem cell-derived cardiomyocyte, an endothelial progenitor cell, a circulating angiogenic cell, a neuron, a pericyte, a cancer cell, a hepatocyte, a pancreatic beta cell, a pancreatic islet cell and combinations thereof.

In one particular aspect, the cell is a circulating angiogenic cell (CAC). CACs are pro-angiogenic cell population that fulfills many roles in vascular biology including the formation of new blood vessels during healing. While CACs are a promising tool for 30 treatment of multiple cardiovascular disorders including peripheral ischemia and restoration of damaged or dysfunctional endothelium; harvesting of CACs is challenging due to their scarcity in the blood stream, so only small numbers of CACs can be isolated at any one time. By culturing the CACs in the hydrogel compositions of the present disclosure, prepared using

hydrogel precursor solutions that encourage proliferation of recruited CACs, the above problem of low initial numbers of recruited CACs and limited CAC expansion is addressed.

In one particular aspect, when used with CACs, the hydrogel compositions include 8-arm, 20 kDa poly(ethylene glycol) (PEG) functionalized with norbornene, a MMP degradable crosslinking peptide, and a cell adhesion peptide. Particularly suitable cell adhesion peptides include immobilized RGD-containing peptides, including CRGDS (SEQ ID NO:2), Acetylated-GCYGRGDSPG (SEQ ID NO:31); cyclic {RGD(Fd)C} (SEQ ID NO:33); CRGD-(G)13-PHSRN (SEQ ID NO:29); and CPHSRN-(SG)5-RGD (SEQ ID NO:30). Suitably, the hydrogel compositions include at least about 1 mM cell adhesion peptide, including from about 1 mM to about 4 mM cell adhesion peptide. Further, the hydrogel compositions may include from about 20 mg/mL to about 100 mg/mL PEG concentration.

In some aspects, the hydrogel compositions are prepared to include crosslinking to an extent of at least 35%, including at least 45%, and including from about 35% to about 75%, and including from about 45% to about 50%.

Suitably, the hydrogel compositions for use with CACs including a shear modulus in the range of from about 1.8 kPa to about 12 kPa, including from about 2 kPa to about 12 kPa.

In another aspect, the cell is a human mesenchymal stem cell (hMSC). It has been found that PEG-hydrogel compositions support hMSC adhesion and expansion. Further, these hydrogel compositions offer combinatorial control over substrate stiffness, cell adhesion, and growth factor regulation.

In one particular aspect, when used with hMSC, the hydrogel compositions include 8-arm, 20 kDa poly(ethylene glycol) (PEG) functionalized with norbornene, a MMP degradable crosslinking peptide, and a cell adhesion peptide. Particularly suitable cell adhesion peptides include immobilized RGD-containing peptides, including CRGDS (SEQ ID NO:2), Acetylated-GCYGRGDSPG (SEQ ID NO:31); cyclic {RGD(Fd)C} (SEQ ID NO:33); CRGD-(G)13-PHSRN (SEQ ID NO:29); and CPHSRN-(SG)5-RGD (SEQ ID NO:30). Suitably, the hydrogel compositions include at least about 0.25 mM cell adhesion peptide, including from about 0.25 mM to about 4 mM cell adhesion peptide, and including from about 1 mM to about 4 mM cell adhesion peptide. Further, the hydrogel compositions may include from about 40 mg/mL to about 160 mg/mL PEG concentration.

Further, the hydrogel compositions for use with hMSCs possess a shear modulus of at least 1.8 kPa, including a shear modulus of from about 1.8 kPa to about 33 kPa, and including from about 1.8 kPa to about 10.9 kPa.

In another aspect, the cell includes a human pluripotent stem cell (hPSC) such 5 as, for example, human embryonic stem cells (hESC) and human induced pluripotent stem cells. Similar to hMSC, it has been found that PEG-hydrogel compositions, and particularly, hydrogel compositions including (PEG) functionalized with norbornene, a MMP degradable crosslinking peptide, and a cell adhesion peptide (e.g., cyclic {RGD(Fd)C} (SEQ ID NO:33), offer substrate stiffness control, cell adhesion control, and growth factor regulation, thereby 10 supporting hPSC adhesion and expansion. Suitably, the hydrogel compositions include at least about 0.25 mM cell adhesion peptide, including from about 0.25 mM to about 4 mM cell adhesion peptide, and including from about 2 mM to about 4 mM cell adhesion peptide.

Further, the hydrogel compositions for use with hPSCs possess a shear modulus of at least 3 kPa, including a shear modulus of from about 3 kPa to about 16 kPa, 15 and including from about 3 kPa to about 10 kPa.

In a further aspect, the hydrogel compositions further include immobilized low molecular weight heparin. Suitably, when present, the hydrogel composition includes low molecular weight heparin in amounts ranging from about 0.1 mM to about 2 mM.

The method may further include contacting the cell with a soluble molecule by 20 including the soluble molecule in the culture medium in which the cells are cultured. Particularly suitable soluble molecules can be growth factors and proteoglycans. Suitable growth factors can be, for example, proteins from the transforming growth factor beta superfamily, fibroblast growth factor family of growth factors, platelet derived growth factor family of growth factors and combinations thereof. Particularly suitable growth factors can 25 be, for example, vascular endothelial growth factor, bone morphogenetic proteins, fibroblast growth factor, insulin-like growth factor and combinations thereof. Suitable proteoglycans and be, for example, proteoglycans with heparin, heparin sulfate, and/or chondroitin glycosaminoglycan side chains.

The disclosure will be more fully understood upon consideration of the 30 following non-limiting Examples.

EXAMPLESMaterials and Methods

PEG-norbornene synthesis

Eight-arm poly(ethylene glycol) (PEG) with terminal hydroxyl groups (-OH) and a molecular weight of 20 kDa was purchased from JenKem Technology USA (Allen, TX). Anhydrous pyridine, 4-dimethylamino)pyridine (DMAP), 5-norbornene-2-carboxylic acid, diethyl ether, and deuterated chloroform ($CDCl_3$, 99.8%) with 0.03 % v/v tetramethylsilane (TMS) were purchased from Sigma Aldrich (St. Louis, MO). N,N'-Dicyclohexylcarbodiimide (DCC) and anhydrous dichloromethane (DCM) were purchased from ACROS Organics (Geel, Belgium). SNAKESKIN dialysis tubing having a 3.5K molecular weight cut-off was purchased from Thermo Fisher Scientific (Waltham, MA).

Eight-arm PEG-OH was functionalized with norbornene to utilize the thiolene chemistry for photopolymerization and immobilization of bioactive ligands (as described in Fairbanks et al. *Adv. Mater.* 2009, 21:5005-5010; Impellitteri et al. *Biomaterials* 2012, 33:3475-84; Belair and Murphy *Acta Biomater.* 2013; and Gould et al. *Acta Biomater.* 2012, 8:3201-3209). The PEG-norbornene (PEG-NB) product of the functionalization reaction was filtered through a medium fritted Buchner funnel to remove salts formed during the reaction. The filtrate was then precipitated in 900 mL cold diethyl ether and 100 mL hexane. The solids were collected on qualitative grade filter paper and air dried overnight. The PEG-NB product was purified by dialysis against 4L of dH_2O at 4°C for 72 hours (with water change every 8 hours) using rehydrated SNAKESKIN dialysis tubing to remove residual norbornene acid and subsequently freeze dried.

Norbornene functionalization of >90% was confirmed with 1H nuclear magnetic resonance spectroscopy. Samples were prepared at 6 mg/mL in $CDCl_3$ with TMS internal standard. Free induction decay (FID) spectra were obtained using spectroscopy services provided by the National Magnetic Resonance Facility at Madison on a Bruker Instruments Avance III 500i spectrometer at 400 MHz and 27°C.

Hydrogel Array Formation

Hydrogel arrays used for these experiments were composed of hydrogel spots immobilized on silanized glass substrates. Hydrogel spots were formed using gold surfaces patterned to possess regions with differential wettability, whereby the pattern was defined by an elastomeric stencil. The method of preparing the hydrogel arrays is further described below.

Glass Silanization

Glass coverslips and hydrochloric acid (HCl) solution were purchased from Thermo Fisher Scientific (Waltham, MA). Toluene, methanol, ethanol, 3-mercaptopropyl trimethoxysilane (3-MPTS), and dithiothreitol (DTT) were purchased from Sigma Aldrich (St. Louis, MO). A low pressure plasma system was purchased from Diener Electronic (Ebhausen, Germany).

Glass coverslips were silanized with 3-MPTS to create substrates presenting thiol groups capable of participating in thiol-ene reaction with PEG-NB and subsequently enable covalent immobilization of PEG-NB hydrogels (Seo et al. *Colloids Surf B Biointerfaces* 2012, 98:1-6). Liquid-phase silanization was performed as previously described (Seo et al. *Colloids Surf B Biointerfaces* 2012, 98:1-6; Halliwell et al. *Anal Chem* 2001, 73:2476-2483; and Cras et al. *Biosens Bioelectron* 1999, 14:683-688). Coverslips were sonicated for 45 minutes in 1:1 methanol to HCl to remove bulk contaminants. Immediately prior to silanization, coverslips were activated by oxygen plasma treatment at 40 sccm and 50 W for 5 minutes on each side to increase the number of activated hydroxyl groups on the surface. Activated coverslips were placed in a coplin jar containing 2.5% v/v 3-MPTS in toluene for 4 hours. Excess silanes were removed from the surface of the coverslips by rinsing with toluene, 1:1 ethanol/ toluene, and ethanol and dried with N₂ gas. Silanized coverslips were placed in an airtight chamber, purged with N₂ gas, and cured at 100°C for 1 hour to crosslink the silanes coupled to the surface and reduce their susceptibility to hydrolysis. Silanized coverslips were stored in the N₂ gas purged chamber and protected from light until use. Prior to use, silanized glass coverslips were treated with 10 mM DTT in PBS for 30 minutes at 37°C to reduce disulfides formed on the surface and to increase free thiols available at the surface (Vistas et al. *Appl Surf Sci* 2013, 286:314-318).

25 Fabrication of Elastomeric Stencils

Silicon wafers were purchased from WRS Materials (San Jose, CA). SU-8 100 photoresist was purchased from MicroChem (Newton, MA). Sylgard 184 silicone elastomer kit was purchased from Dow Corning Corporation (Midland, MI).

Polydimethylsiloxane (PDMS) elastomeric stencils were created using soft lithography as previously described (Jo et al. *J Microelectromechanical Syst* 2000, 9:76-81). The layout and geometries for the stencil were drawn using Adobe Illustrated, printed onto transparency films using a high resolution commercial laser printing service provided by ImageSetter (Madison, WI). The transparency film was used as a photo mask in combination

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with conventional photolithography techniques to create master molds with SU-8 negative-tone UV photoresist spin-coated on silicon wafers. To create the PDMS stencil, the curing agent and PDMS pre-polymer solution from the Sylgard elastomer kit were thoroughly mixed in a 1:10 weight ratio, spread onto the master mold, and cured at 80°C for 6 hours. After 5 curing, the PDMS stencils were peeled off from the master mold, briefly cleaned with ethanol, and dried with N₂ gas.

Hydrophobic/Hydrophilic Patterning

Gold-coated test slides (1,000 Å gold on 50 Å titanium metal thin films on 25 mm x 75 mm x 1 mm glass) were purchased from Evaporated Metal Films (Ithica, NY). 10 Perfluorinated alkanethiol (HS-(CH₂)₁₁-O-(CH₂)₂-(CF₂)₅-CF₃) was purchased from ProChimia Surfaces (Sopot, Poland). Hydroxyl-terminated alkanethiol (HS-C₁₁-(O-CH₂-CH₂)₃-OH) was synthesized as previously described (Prime and Whitesides J. Am. Chem. Soc. 1993, 115:10714-10721).

Gold-coated slides were patterned with hydrophobic and hydrophilic self-assembled monolayers (SAMs) of alkanethiolates to form regions with differential wettability. Differential wettability patterning served two purposes simultaneously: 1) defined the geometries of the hydrogel spots and 2) confined the contents of each hydrogel spot in the array. Gold-coated slides were immersed in ethanol and sonicated for ~2 minutes, rinsed with ethanol, and dried with N₂ gas to remove contaminants and gold oxide layers. 15 Gold-coated slides were immersed in a 1 mM perfluorinated alkanethiol in ethanol solution for ≥2 hours to allow for perfluorinated alkanethiolate SAMs (fluoraSAMs) formation. After fluoraSAMs formation, fluoraSAMs gold-coated slides were cleaned with ethanol and dried with N₂ gas. To define hydrophilic regions on the substrate, PDMS stencils were placed on the fluoraSAMs gold-coated slides to selectively protect areas of the slides from plasma 20 etching. The spatial and geometric patterning of the exposed regions on the fluoraSAMs gold-coated slides were defined by the pattern of the PDMS stencil, which, in turn, defined the geometry and spatial patterning of the hydrogel spots that the arrays could comprise. Exposed regions of the fluoraSAMs gold-coated slides were etched by oxygen plasma treatment at 40 sccm and 50 W for 1 minute. The etched gold-coated slides were cleaned 25 with ethanol and dried with N₂ gas and immersed in a 0.1 mM hydroxyl-terminated alkanethiol in ethanol solution for ≥2 hours so that hydrophilic alkanethiolate SAMs (EG₃SAMs) were formed in the selectively-etched regions of the gold-coated slides. The

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resulting gold-coated slides with differential wettability were cleaned with ethanol and dried with N₂ gas before hydrogel formation.

Hydrophobic and hydrophilic SAMs formation on the gold-coated slides were confirmed with contact angle measurements (see, FIG. 2B). Static contact angles were 5 measured at room temperature using a contact angle goniometer (DataPhysics Contact Angle System OCA, San Jose, CA). A drop of distilled water (3 μ L) was placed on the surface and the static contact angle was measured for 3 different samples at five different sites on each sample and averaged.

Hydrogel Spot Polymerization and Immobilization

10 PEG-NB was functionalized as described above. Bi-functional PEG dithiol (PEG-DT) crosslinker (3.4 kDa) was purchased from Laysan Bio (Arab, AL). IRGACURE 2959 photoinitiator was purchased from Ciba/BASF (Ludwigshafen, Germany). Cysteine-terminated peptides were purchased from GenScript USA (Piscataway, NJ). Omnicure Series 1000 UV spot cure lamp (365 nm wavelength), light guide, and collimating adapter were 15 purchased from Lumen Dynamics Group (Ontario, Canada). PDMS spacers with thickness dimensions corresponding to the desired hydrogel spot heights were fabricated using the same procedure as stated above.

20 Hydrogel precursor solutions were prepared by combining PEG-NB, PEG-DT, peptides, and photoinitiator and diluted to desired concentrations with phosphate buffered saline (PBS) immediately prior to hydrogel spots formation. To form each hydrogel array, a patterned gold-coated slide was rinsed with ethanol and dried with N₂ gas, PDMS spacers were placed onto hydrophobic regions of the slide, and hydrogel precursor solutions were 25 spotted onto the hydrophilic regions. A DTT-treated silanized glass coverslip was used to sandwich the hydrogel precursor solutions between the coverslip and the slide. Hydrogel precursor solutions were polymerized by UV-initiated photo-crosslinking for 2 seconds at 90 mW/cm², with the light penetrating through the glass coverslip. The resulting polymerized hydrogel spots were covalently attached and immobilized onto the coverslip. Recall that the 30 silanization procedure produced glass coverslips that were functionalized with thiol-terminated silanes that were capable of participating in the thiol-ene reaction used for hydrogel precursor solution polymerization, which effectively crosslinked the hydrogel network to the surface-bound silanes. The gold-coated slide was separated from the coverslip, which enabled the glass-immobilized hydrogel spots to cleanly detach from the gold-coated slide. The resulting glass-immobilized hydrogel spots, collectively referred to as

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the “hydrogel array”, was sterilized for 1 hour in 70% ethanol and washed with PBS to remove any remaining unreacted components.

The bioactivity of each hydrogel spot in the array was defined by both the identity and concentration of the peptides incorporated therein. Peptides used in this study 5 were CRGDS (SEQ ID NO:2), CRGD-(G)₁₃-PHSRN (SEQ ID NO:29), CRGD-(SG)₅-PHSRN (SEQ ID NO:30), acetylated-CRGDSP (SEQ ID NO:31), cyclic (RGD{Fd}C) (SEQ ID NO:33), and a non-bioactive scrambled peptide CRDGS (SEQ ID NO:32). To modulate the bioactivity of each hydrogel spot, different peptides were added to the hydrogel precursor 10 solutions and, following UV-initiated crosslinking, the resulting polymerized hydrogel networks each presented different immobilized peptides. For all arrays, a total of 4 mM of peptides were incorporated into the hydrogel network. To concurrently change the bioactivity of the hydrogel spots via control of peptide identity and concentration, the desired concentration of the chosen bioactive peptide (containing the “RGD” sequence) was determined and the CRDGS (SEQ ID NO:32) peptide was supplemented to maintain a total 15 peptide concentration of 4 mM in the hydrogel precursor solution.

The modulus of each hydrogel spot in the hydrogel array was defined by the total concentration of PEG in the crosslinked hydrogel network. Increasingly, the concentration of PEG-NB in the hydrogel precursor solution resulted in a larger amount of PEG crosslinked into the polymerized network, which resulted in an increase in the 20 compressive modulus (see, FIG 8).

EXAMPLE 1

In this Example, a hydrogel array immobilized on a glass substrate was prepared.

A gold substrate was modified with a patterned alkanethiolate self-assembled monolayer (SAMs) to provide isolated hydrophilic regions separated by a surrounding 25 hydrophobic region (as illustrated in FIGS. 1A-1B). As illustrated in FIG. 2A (also shown in FIG. 1A), hydrophobic and hydrophilic SAMs formation on the gold-coated slides were confirmed with contact angle measurements. FIG. 2B provides end views during patterning of a gold substrate at the step before hydrophobic patterning 100; of the substrate having fluoraSAMs 110; of the substrate after etching 120; and of the substrate after hydrophilic 30 patterning 130.

Hydrogel precursor solutions containing all components required for polymerization reactions were deposited onto the hydrophilic SAMs regions of the patterned

substrate (see, FIG. 1B). The hydrophilic regions served to both confine the contents of the solutions deposited onto each region and to define the geometries of the resulting polymerized hydrogel. Elastomeric spacers (with thickness dimensions equivalent to the desired hydrogel array height) were placed onto the hydrophobic areas of the patterned slide 5 to define the height of the hydrogel array. A glass substrate, modified by silanization to possess SAMs with end-functional groups capable of participating in the polymerization reaction, was used to sandwich the hydrogel precursor solution. During the UV polymerization, the components of the hydrogel precursor solution formed a crosslinked network as well as formed covalent bonds with the end-function groups on the glass 10 substrate. The polymerized hydrogels removed cleanly from the patterned gold substrate to produce a hydrogel array immobilized on the glass substrate (see, FIG. 3).

EXAMPLE 2

In this Example, a hydrogel array was used to determine the effects of substrate properties on initial stem cell adhesion.

Poly (ethylene glycol) (PEG) hydrogel arrays were formed using patterned 15 hydrophobic/hydrophilic self-assembled monolayers on gold substrates to both define the geometry and confine the contents of each hydrogel spot in the array as described above (see, FIGS. 1A-1B). UV-initiated thiol-ene crosslinking simultaneously polymerized the hydrogel and immobilized the hydrogel spots on the glass to result in the hydrogel array. As illustrated in FIG. 9, hydrogel arrays could be prepared with dimensions compatible with a 64-well 20 microarray add-on (commercially available from Grace Bio-Labs, Bend, OR).

Hydrogel solutions with fibronectin-derived peptides, fluorescent microspheres and a dithiol crosslinker were deposited onto the SAMs and sandwiched with a silanized glass slide. As shown in FIG. 7, individual hydrogel spots of the hydrogel array 25 could be prepared to include varying amounts of fluorescently-tagged peptides as well as varying amounts of fluorescent microspheres. Hydrogel solutions with varying PEG or crosslinker concentration were also prepared prior to crosslinking to change the stiffness, peptide identity or peptide concentration (FIG. 8). The resultant arrays (see, FIG. 3) included 2.4 mm diameter, 150 um height posts. Human mesenchymal stem cells (hMSCs) were cultured on posts with varying PEG concentrations (4 wt %, 6 wt % and 8 wt %) to change 30 stiffness and monitored for changes in initial cell adhesion and spreading. Human embryonic stem cells (hESCs) were cultured on posts with varying peptide identity (blank, RDGS,

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RGDS (SEQ ID NO:1), RGD-PHSRN (SEQ ID NO:34), RGDSP (SEQ ID NO:47), and cyclic RGD) and monitored for changes in initial cell adhesion and spreading.

As shown in FIGS. 10A-10C, 2D culture of hMSCs demonstrated cell spreading dependence in response to changes in modulus consistent with published 5 observations (see, Engler et al. Cell 126:677 (2006)). 2D culture of hESCs in chemically-defined, albumin-free media demonstrated that cell adhesion was highly specific to peptide-presenting spots. Both hESC cell adhesion and spreading were dependent on the binding affinity of integrin receptors to immobilized peptides (see, FIG. 11). Arrays allowed for changes in hydrogel spot shape, hydrogel spot height (by changing patterned hydrogel spot 10 shapes or adding spacers), hydrogel spot stiffness and hydrogel spot peptide concentrations, and was adaptable for both 2D and 3D cell culture.

These results demonstrate that the method for preparing hydrogel arrays as described herein provides the capability to control stiffness, immobilized ligand identity and ligand concentration (density), and soluble growth factor presentation. The hydrogel arrays 15 of the present disclosure can support cell adhesion and survival and allow for screening complex cell-environment interactions.

EXAMPLE 3

In this Example, the hydrogel compositions of the present disclosure were prepared, human mesenchymal stem cells (hMSCs) were cultured thereon, and cell properties were analyzed.

20 Hydrogel arrays containing hydrogel spots with stiffness values ranging from 1.8 to 10.9 kPa and varying immobilized CRGDS (SEQ ID NO:2) peptide concentrations from 0 to 4 mM were prepared as described herein. Note that total peptide concentration was maintained at 4 mM by supplementing the hydrogel precursor solution with a “scrambled”, non-bioactive CRDGS peptide (SEQ ID NO:32). The stiffness range selected for this screen 25 was chosen to reflect the reported stiffness values of various soft tissues, including fat and muscle tissue. Following hydrogel array formation, hMSCs were seeded onto the hydrogel spots and cultured for up to 8 days.

Independent of hydrogel spot stiffness, hMSC initial cell attachment, spreading, and proliferation were linearly correlated with immobilized CRGDS (SEQ ID 30 NO:2) concentration. After 1 day of culture, minimal hMSCs were attached to hydrogel spots that did not contain CRGDS (SEQ ID NO:2) (4 mM “scrambled” CRDGS (SEQ ID NO:32)), indicating that initial cell adhesion was mediated by the bioactivity of the

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immobilized peptide. Increased CRGDS (SEQ ID NO:2) concentration resulted in increased hMSC cell attachment, spreading, and proliferation with maximal values on hydrogel spots presenting 4 mM CRGDS (SEQ ID NO:2).

Increasing hydrogel spot stiffness (from 1.8 to 10.9 kPa) also resulted in 5 increased hMSC initial cell attachment, spreading, and proliferation. Maximal hMSC initial cell attachment was seen on hydrogel spots of 8.2 kPa stiffness while maximal cell spreading and proliferation were both seen on hydrogel spots of 5.4 kPa stiffness. Results are shown in FIGS. 12-14.

EXAMPLE 4

10 In this Example, hydrogel compositions of the present disclosure were prepared, human embryonic stem cells (hESCs) were cultured thereof, and cell expansion was analyzed.

15 Hydrogel arrays containing hydrogel spots with stiffness values ranging from 1.8 to 10 kPa and varying immobilized cyclic RGD{Fd}C peptide (SEQ ID NO:33) concentrations from 0 to 4 mM. Note that total peptide concentration was maintained at 4 mM by supplementing the hydrogel precursor solution with a “scrambled”, non-bioactive cyclic-RAD{d-Phe} peptide (SEQ ID NO:48). Following hydrogel array formation, H1 hESCs were seeded onto the hydrogel spots and cultured for up to 5 days. H1 hESCs cultured on hydrogels of 3-10 kPa stiffness and containing 2 – 4 mM cyclic-RGD{d-Phe} 20 peptide exhibited cell spreading and proliferation rates similar to those cultured on MATRIGEL®-coated tissue culture polystyrene. Results are shown in FIGS. 15 & 16.

CLAIMS

We claim:

1. A method of promoting cellular expansion, the method comprising:
 - preparing a hydrogel composition, wherein the hydrogel composition comprises a polyethylene glycol functionalized with norbornene, a crosslinking peptide, and a cell adhesion peptide;
 - contacting a cell the hydrogel composition; and
 - culturing the cell.
2. The method of claim 1 wherein the polyethylene glycol functionalized with norbornene comprises an 8-arm, 20 kDa polyethylene glycol functionalized with norbornene.
3. The method of claim 1 wherein the cell is a circulating angiogenic cell.
4. The method of claim 3 wherein the hydrogel composition comprises at least 1 mM cell adhesion peptide selected from the group consisting of CRGDS (SEQ ID NO: 2), Acetylated-GCYGRGDSPG (SEQ ID NO:31), cyclic RGD (SEQ ID NO:35), CRGD-(G)₁₃-PHSRN (SEQ ID NO:29), and CPHSRN-(SG)₅-RGD (SEQ ID NO:30).
5. The method of claim 3 wherein the hydrogel composition comprises a shear modulus range of from about 2 kPa to about 12 kPa.
6. The method of claim 1 wherein the cell is a human mesenchymal stem cell.
7. The method of claim 6 wherein the hydrogel composition comprises a shear modulus of from about 1.8 kPa to about 33 kPa.
8. The method of claim 6 wherein the hydrogel composition comprises at least 0.25 mM of cell adhesion peptide selected from the group consisting of CRGDS (SEQ ID NO: 2), Acetylated-GCYGRGDSPG (SEQ ID NO:31), cyclic RGD (SEQ ID NO:35), CRGD-(G)₁₃-PHSRN (SEQ ID NO:29), and CPHSRN-(SG)₅-RGD (SEQ ID NO:30).
9. The method of claim 6 wherein the cell is a human pluripotent stem cell selected from the group consisting of a human embryonic stem cell and human induced pluripotent stem cell.
10. The method of claim 9 wherein the hydrogel composition comprises at least 0.25 mM of cell adhesion peptide selected from the group consisting of CRGDS (SEQ ID NO: 2),

Acetylated-GCYGRGDSPG (SEQ ID NO:31), cyclic RGD (SEQ ID NO:35), CRGD-(G)₁₃-PHSRN (SEQ ID NO:29), and CPHSRN-(SG)₅-RGD (SEQ ID NO:30).

11. The method of claim 9 wherein the hydrogel composition comprises a shear modulus of from about 3 kPa to about 16 kPa.

12. The method of claim 9 wherein the hydrogel composition further comprises immobilized low molecular weight heparin.

13. A method of promoting cellular differentiation, the method comprising:
preparing a hydrogel composition, wherein the hydrogel composition comprises a polyethylene glycol functionalized with norbornene, a crosslinking peptide, and a cell adhesion peptide;

contacting a cell with the hydrogel composition; and
culturing the cell.

14. The method of claim 13 wherein the polyethylene glycol functionalized with norbornene comprises an 8-arm, 20 kDa polyethylene glycol functionalized with norbornene.

15. The method of claim 13 wherein the cell is a human mesenchymal stem cell.

16. The method of claim 15 wherein the hydrogel composition comprises a shear modulus of from about 1.8 kPa to about 33 kPa.

17. The method of claim 15 wherein the hydrogel composition comprises at least 0.25 mM of cell adhesion peptide selected from the group consisting of CRGDS (SEQ ID NO: 2), Acetylated-GCYGRGDSPG (SEQ ID NO:31), cyclic RGD (SEQ ID NO:35), CRGD-(G)₁₃-PHSRN (SEQ ID NO:29), and CPHSRN-(SG)₅-RGD (SEQ ID NO:30).

18. The method of claim 13 wherein the cell is a human pluripotent stem cell selected from the group consisting of a human embryonic stem cell and human induced pluripotent stem cell.

19. The method of claim 18 wherein the hydrogel composition comprises at least 0.25 mM of cell adhesion peptide selected from the group consisting of CRGDS (SEQ ID NO: 2), Acetylated-GCYGRGDSPG (SEQ ID NO:31), cyclic RGD (SEQ ID NO:35), CRGD-(G)₁₃-PHSRN (SEQ ID NO:29), and CPHSRN-(SG)₅-RGD (SEQ ID NO:30).

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20. The method of claim 18 wherein the hydrogel composition comprises a shear modulus of from about 3 kPa to about 16 kPa.

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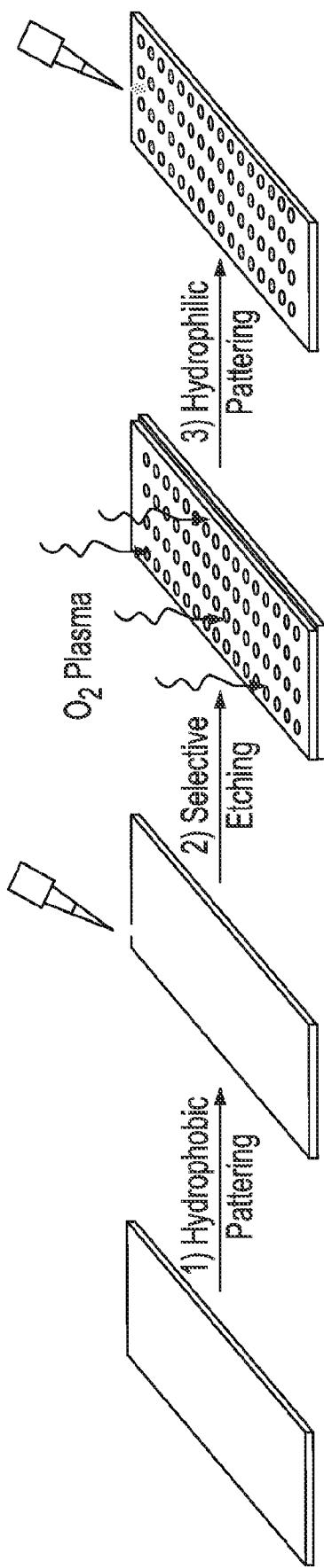


FIG. 1A

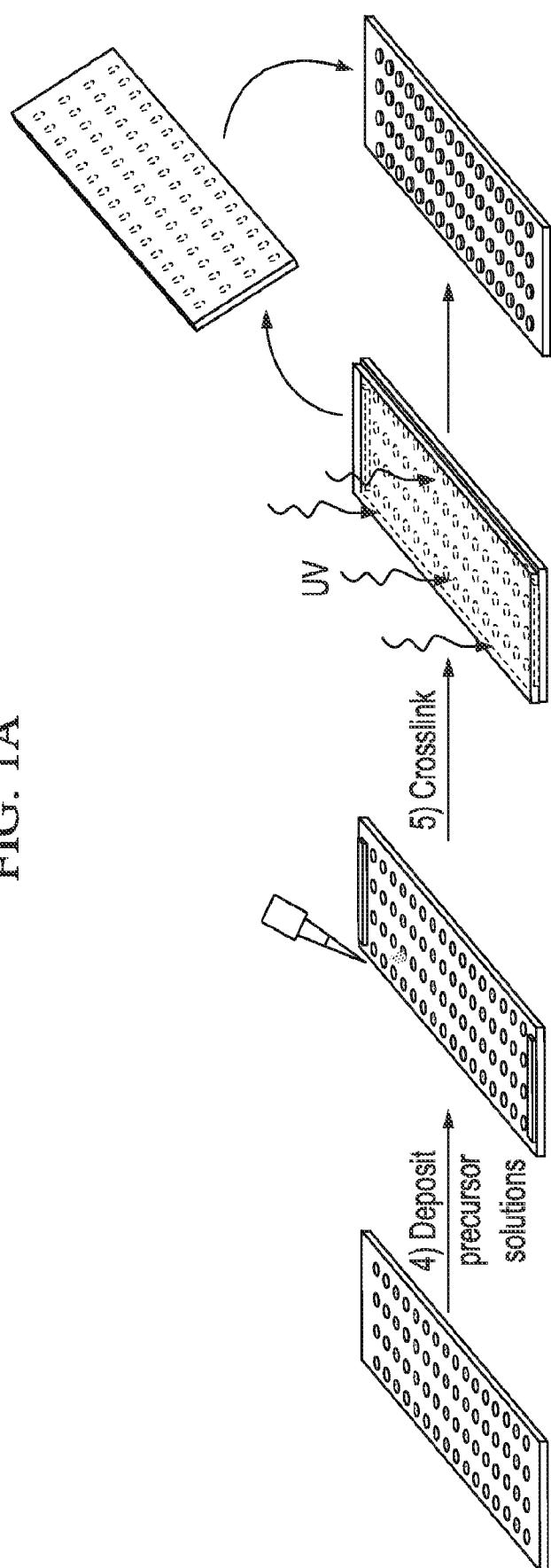


FIG. 1B

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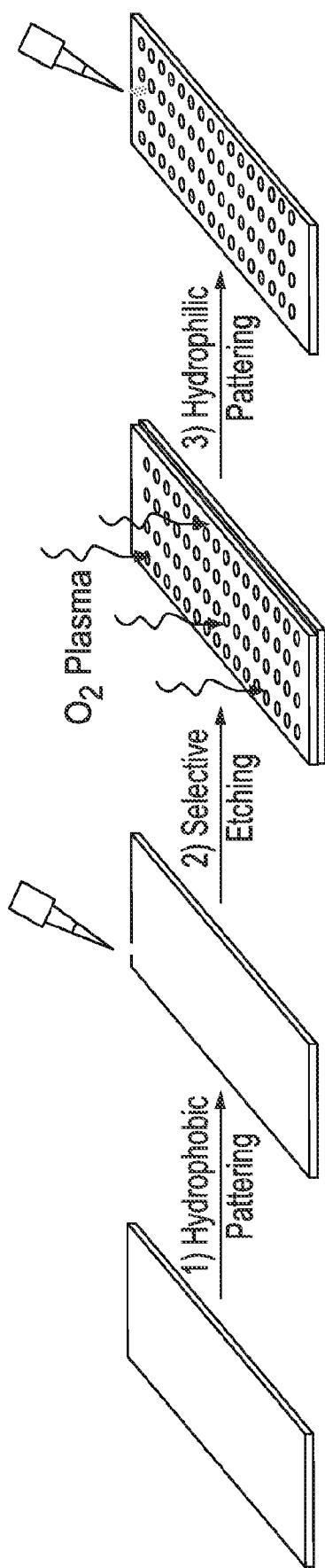


FIG. 2A

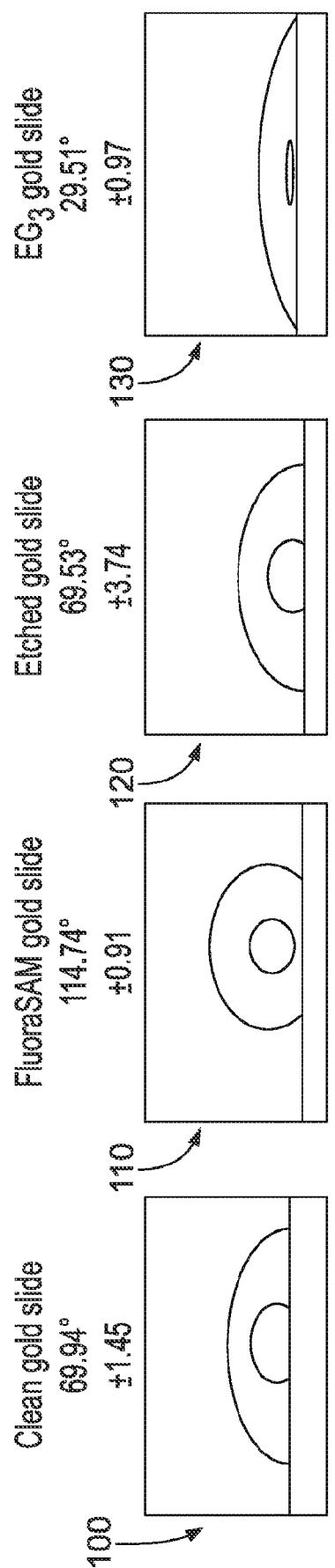


FIG. 2B

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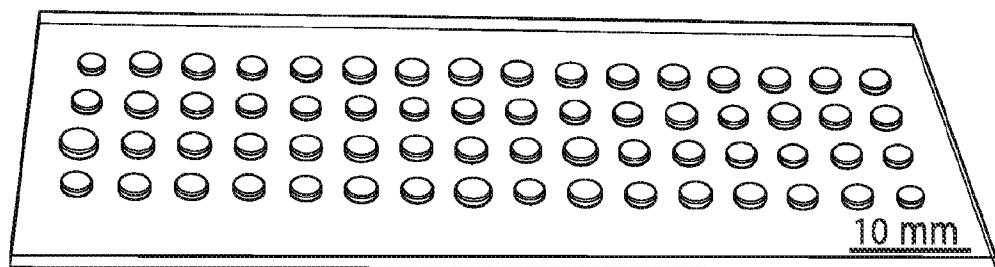


FIG. 3

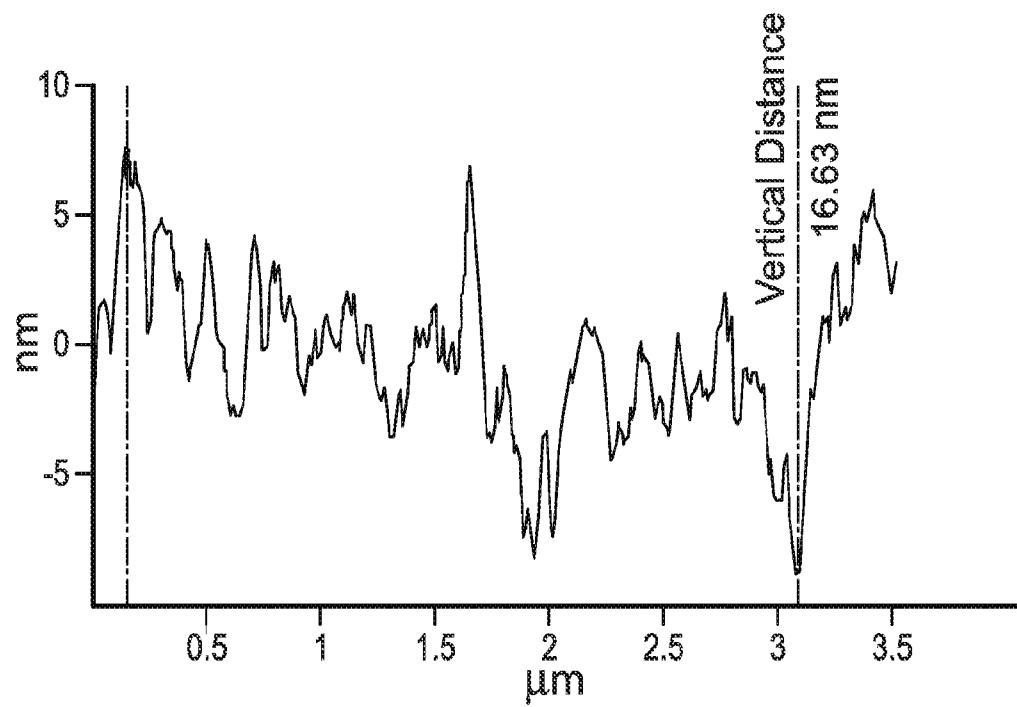


FIG. 4

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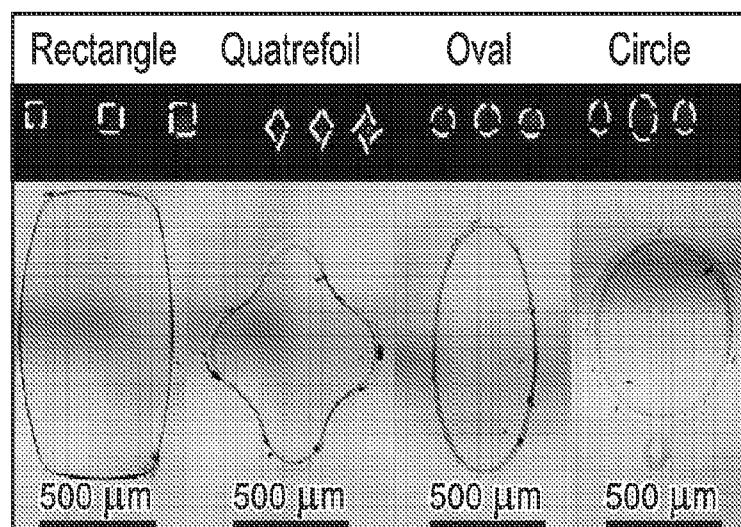


FIG. 5

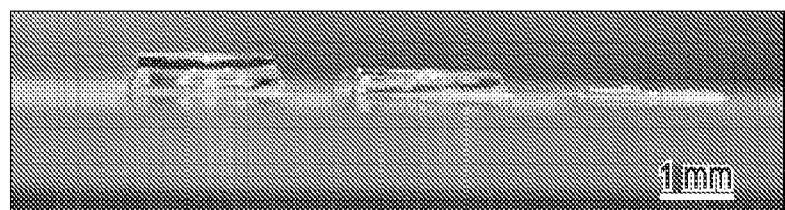


FIG. 6

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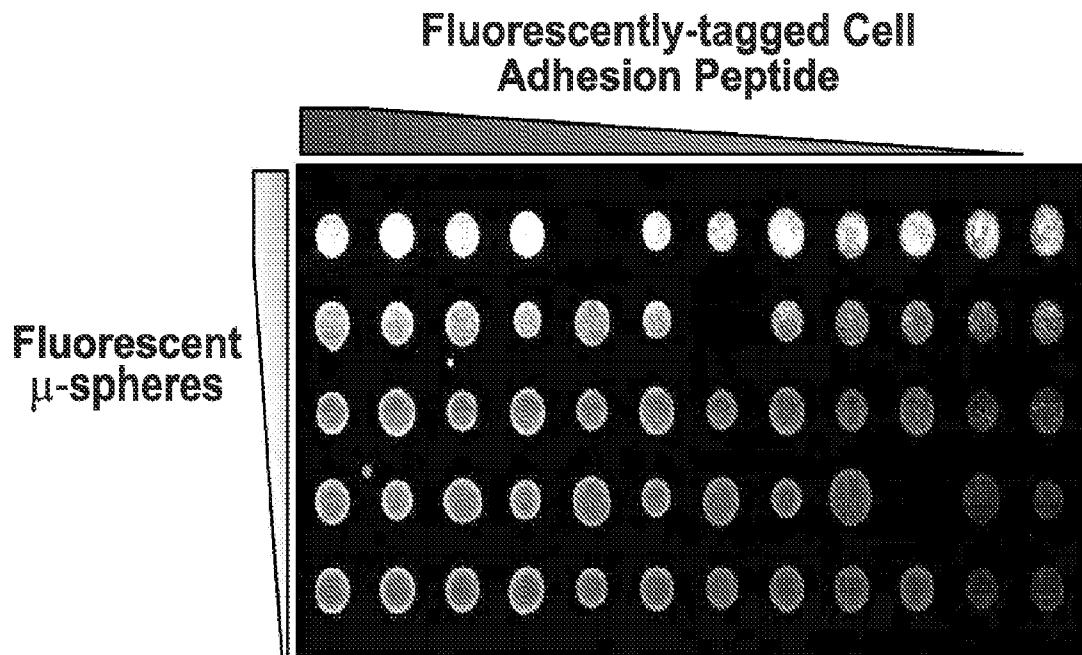


FIG. 7

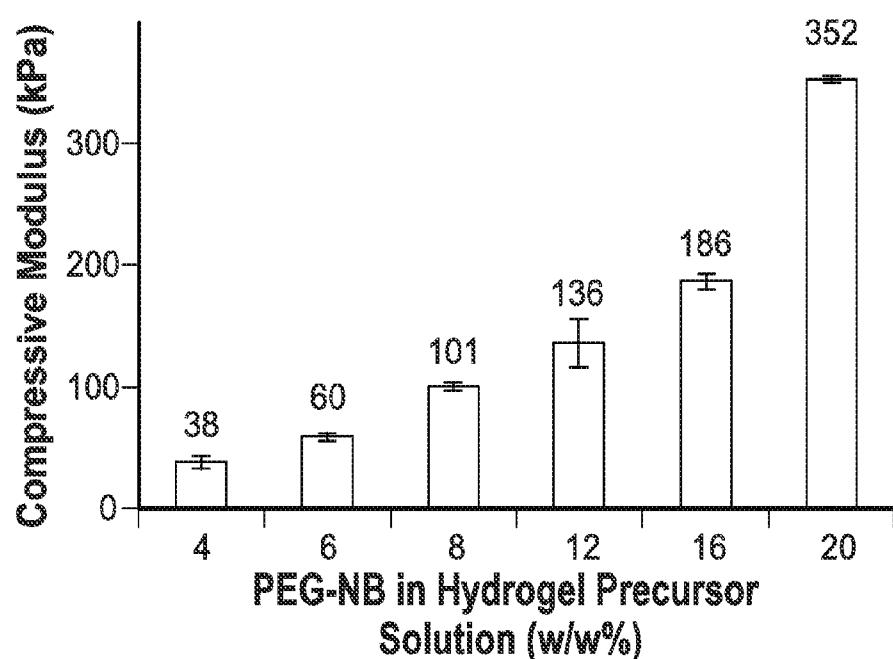
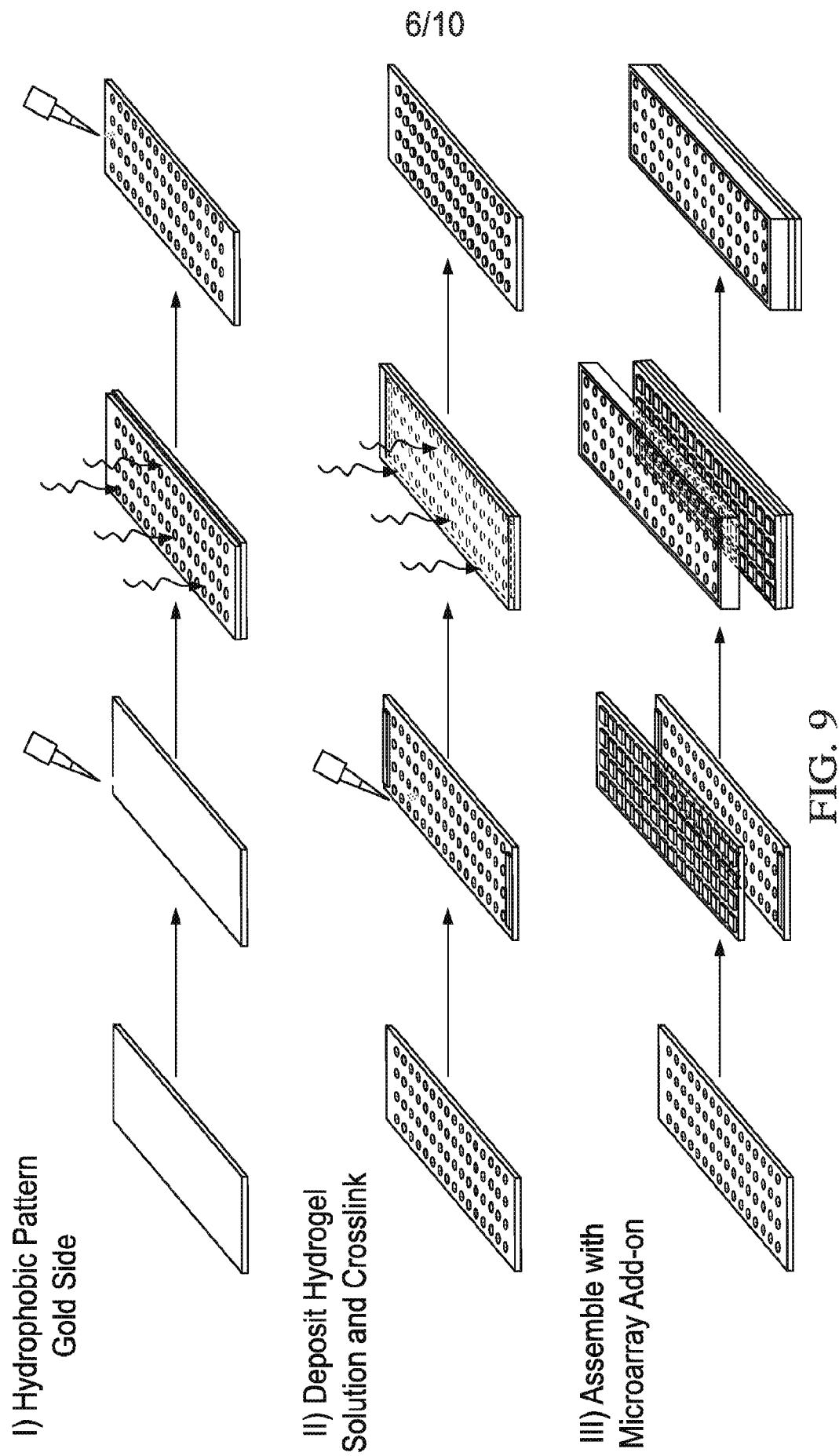


FIG. 8



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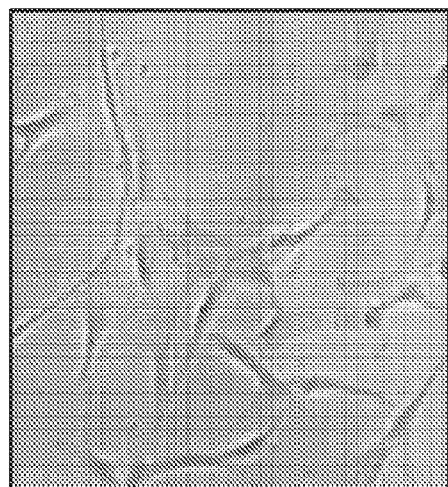


FIG. 10C

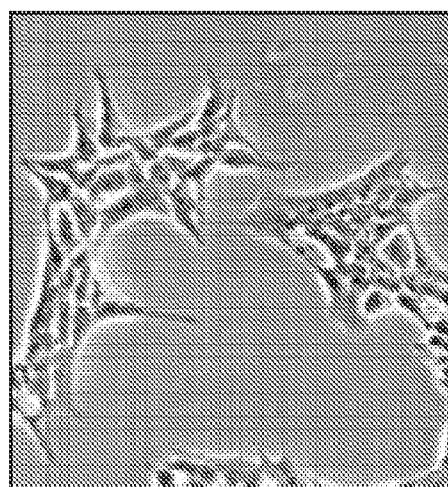


FIG. 11C

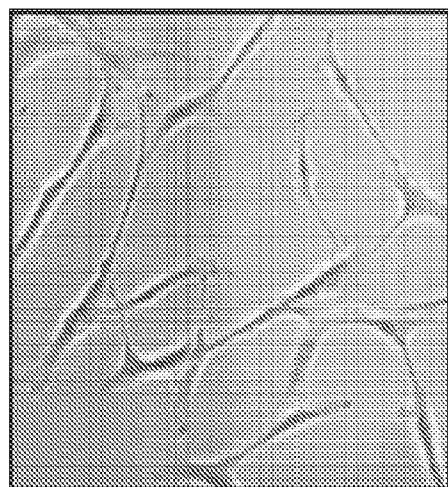


FIG. 10B

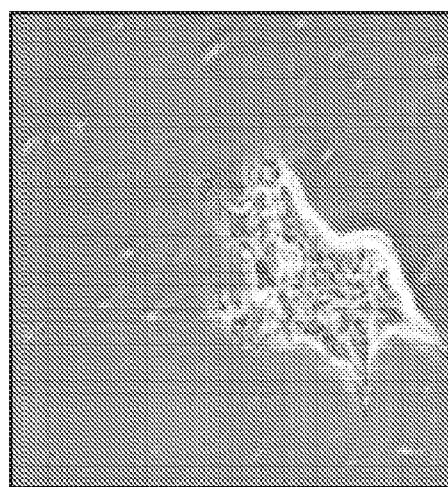


FIG. 11B

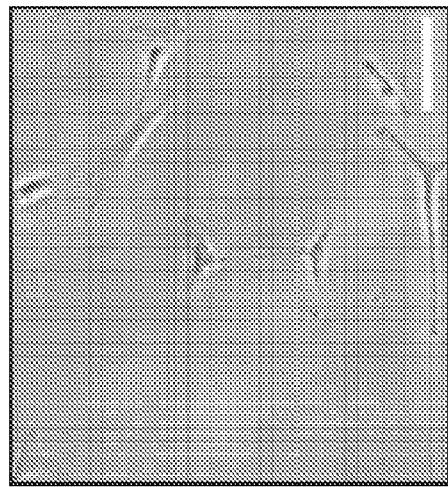


FIG. 10A

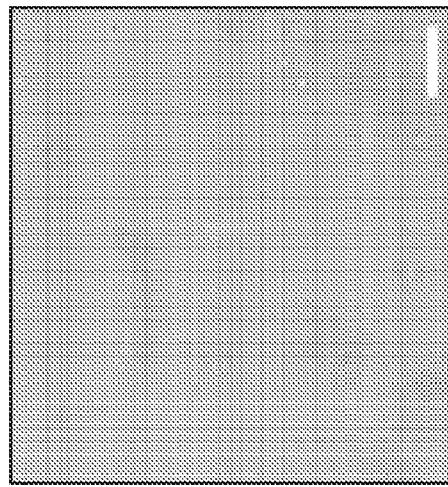


FIG. 11A

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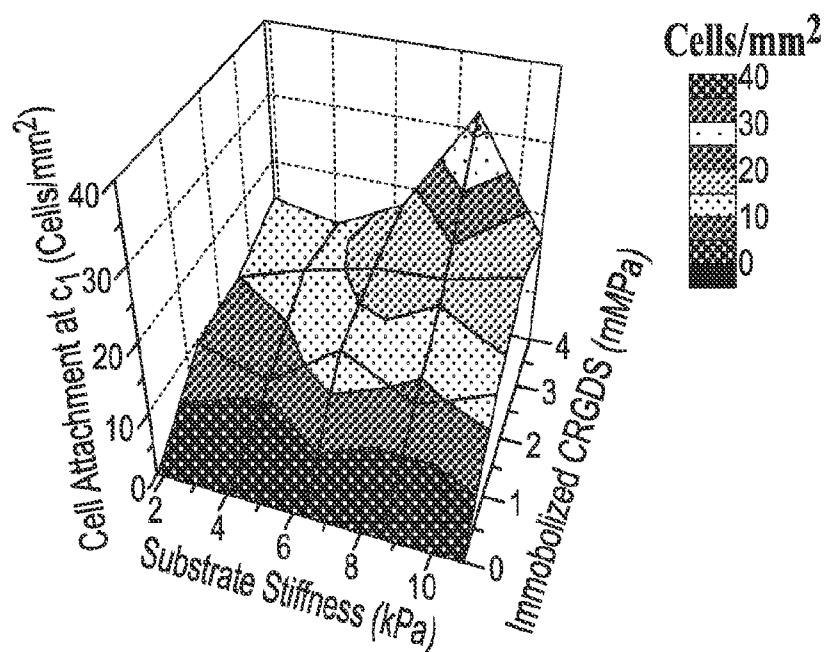


FIG. 12

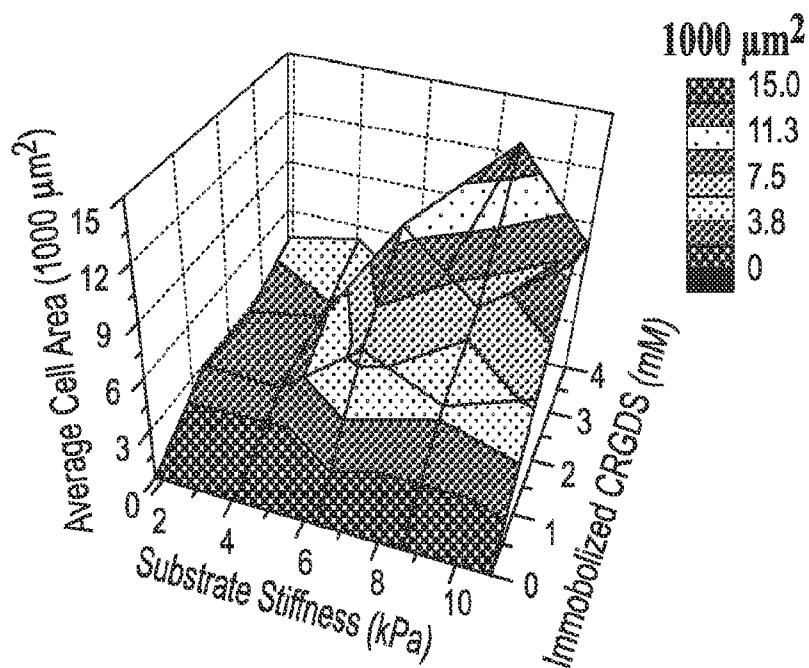


FIG. 13

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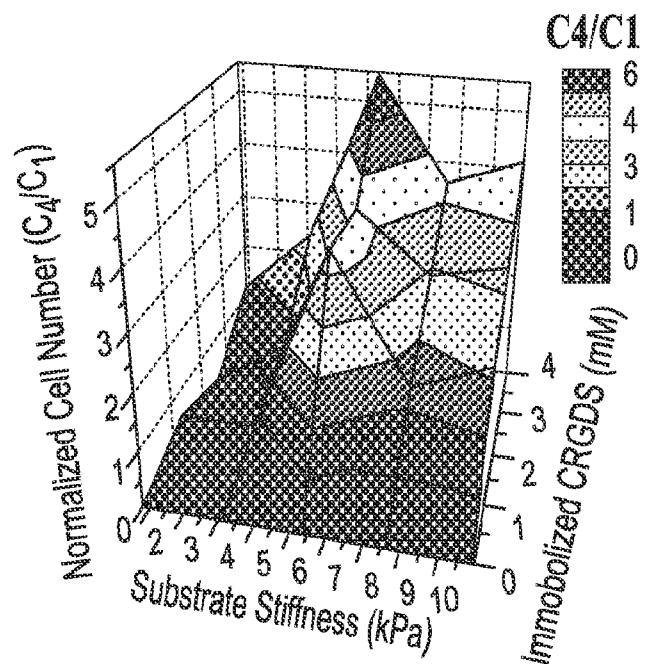


FIG. 14

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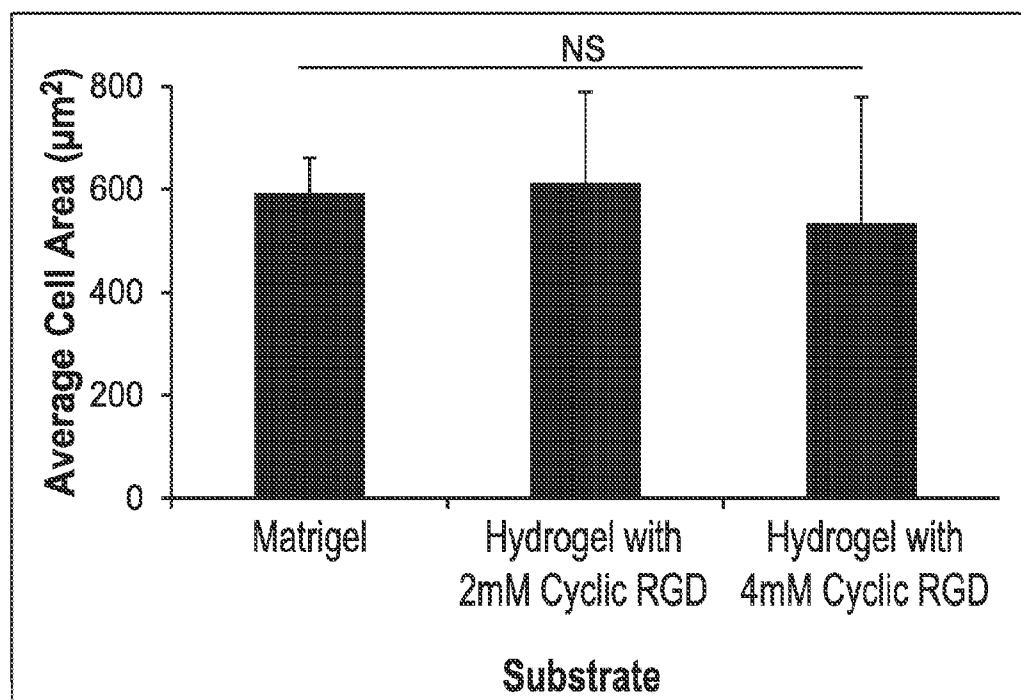


FIG. 15

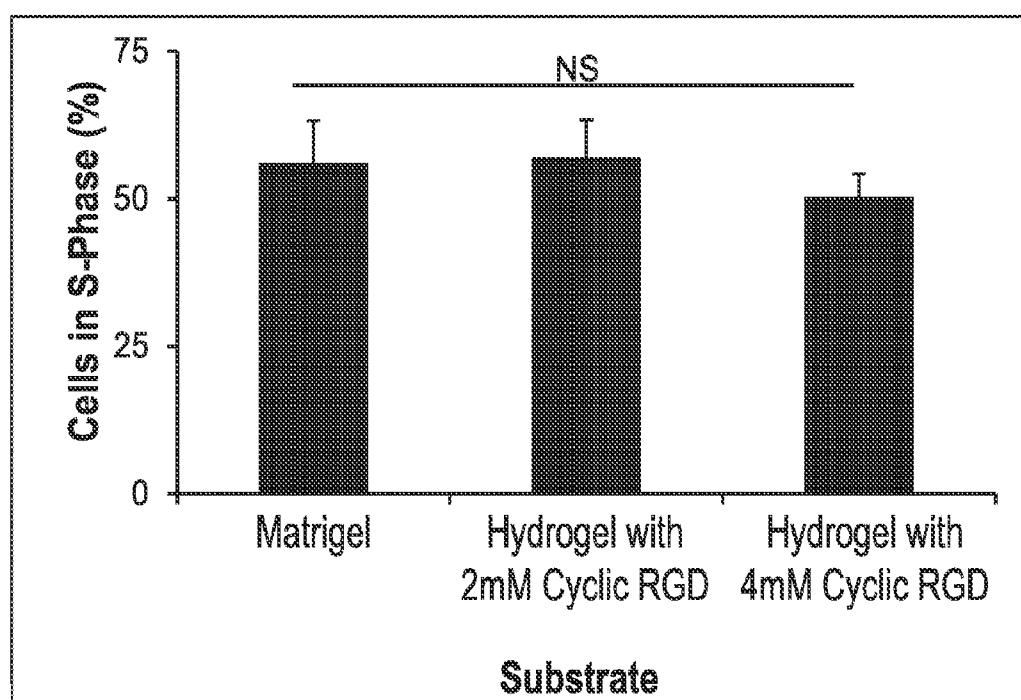


FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/025479

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 5/00; C12N 5/071; A61F 2/02; C12N 5/02; A61K 35/12; A61K 47/42; A61K 38/18Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility modelsElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: hydrogel composition, PEG-NB, cellular expansion, cellular differentiation, RGD-containing peptide, stiffness

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012-0149781 A1 (LEE, KI-BUM et al.) 14 June 2012 See abstract; claims 1-20; and paragraphs [0034]-[0095].	1-20
A	US 2012-0225814 A1 (HANJAYA-PUTRA, DONNY et al.) 06 September 2012 See abstract; claims 1, 3, 7, 38; and pages 6-7.	1-20
A	US 2013-0210147 A1 (JEANNIN, LAURENT et al.) 15 August 2013 See abstract; claims 1-19; pages 3, 5-6; and table 1.	1-20
A	US 2014-0017284 A1 (YANG, FAN et al.) 16 January 2014 See abstract; claims 1-19; and paragraphs [0038]-[0040], [0150].	1-20
A	RAZA, ASAD et al., 'The influence of matrix degradation and functionality on cell survival and morphogenesis in PEG-based hydrogels', Macromolecular Bioscience, 2013, Vol.13, No.8, pp.1048-1058 See the whole document.	1-20
A	BANERJEE, AKHILESH et al., 'The influence of hydrogel modulus on the proliferation and differentiation of encapsulated neural stem cells', Biomaterials, 2009, Vol.30, No.27, pp.4695-4699 See the whole document.	1-20

 Further documents are listed in the continuation of Box C. See patent family annex.

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 "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
26 June 2015 (26.06.2015)

Date of mailing of the international search report

29 June 2015 (29.06.2015)Name and mailing address of the ISA/KR

 International Application Division
 Korean Intellectual Property Office
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 Republic of Korea
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Authorized officer

HEO, Joo Hyung



Telephone No. +82-42-481-8150

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2015/025479

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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US 2012-0225814 A1	06/09/2012	CA 2780490 A1 EP 2504432 A2 EP 2504432 A4 IL 219728 D0 US 8900868 B2 WO 2011-060095 A2 WO 2011-060095 A3	19/05/2011 03/10/2012 02/07/2014 31/07/2012 02/12/2014 19/05/2011 24/11/2011
US 2013-0210147 A1	15/08/2013	EP 2625191 A1 EP 2625192 A1 JP 2013-538864 A US 2013-0281602 A1 WO 2012-045822 A1 WO 2012-045824 A1	14/08/2013 14/08/2013 17/10/2013 24/10/2013 12/04/2012 12/04/2012
US 2014-0017284 A1	16/01/2014	None	