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Abstract

Provided herein are thermoreversible polymers with improved stability, hydrogel compositions comprising the thermoreversible polymers as well as an *in vitro* method for producing a cell population enriched for inhibitory GABAergic cortical interneurons from human pluripotent cells utilizing the hydrogel compositions.

Thermoreversible Polymers with Improved Stability and Methods and Uses Thereof

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application Serial No. 63/595,847, filed November 3, 2023 and U.S. Provisional Patent Application Serial No. 63/595,841, filed November 3, 2023, the entire contents of each of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] Human pluripotent stem cells (hPSCs) have the potential to differentiate into any cell type in the body and therefore have broad applications for regenerative medicine. However, the expansion and differentiation of hPSCs at large scale remains to be a major challenge in the field, preventing these transformative therapies from reaching the large patient population in need. Here we describe the generation of a synthetic, fully defined thermoreversible hydrogel that allows for the growth and differentiation of hPSCs into sensitive cell types at large scale. The improved hydrogel formulation allows for a lower viscosity gel that enables encapsulation of sensitive cell types utilizing a diverse range of liquid handling systems, thanks to these unique properties: formulation and molecular weight (MW) of polymer renders a low viscosity hydrogel ideal for hPSC culture and differentiation into all 3 germ layers. This minimizes shear during a process to encapsulate cells for large scale bioreactor systems. This formulation and MW also allows for faster flow rates during encapsulation and higher control of bead size/geometry. MW of polymer and PEG length is optimal to render stable hydrogels that maintain structure through lengthy differentiation processes and render stiffness range that supports expansion and differentiation of hPSCs into cells in all 3 germ layers. This formulation is also compatible with functionalization, allowing for controlled protein presentation and release. Utilizing this thermoreversible hydrogel we were able to expand hPSCs at large scale and differentiate into several cell fates including midbrain dopaminergic neurons, cortical interneurons, pancreatic endoderm progenitor cells and hematopoietic stem cells.

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SUMMARY OF THE INVENTION

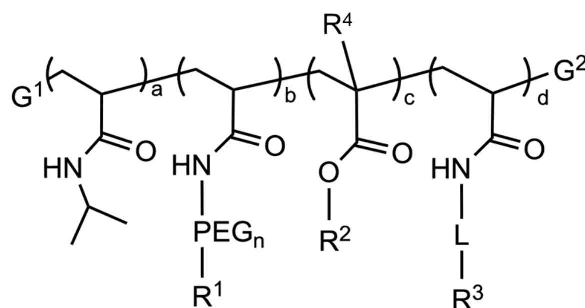
[0003] Provided herein are *in vitro* scalable methods to produce post-mitotic inhibitory GABAergic cortical interneurons (cINs) from human stem cells (e.g., human pluripotent or multipotent cells) comprising encapsulation of the stem cells in a three-dimensional synthetic hydrogel under conditions whereby a population of cells comprising cINs is produced. In some aspects, the pluripotent cell is a human pluripotent stem cell (hPSC) such as a human embryonic stem cell or a human induced pluripotent stem cell (iPSC). Preferably, the method is xeno-free and the hydrogel does not comprise extra cellular matrix (ECM) proteins.

[0004] In preferred embodiments, the three-dimensional hydrogel for use in the methods is a thermoresponsive (e.g., thermoreversible) hydrogel. In some embodiments, the three-dimensional hydrogel is a polyethylene glycol-Poly(N-isopropylacrylamide) (PEG-PNIPAAM) based hydrogel. In some embodiments, the thermoresponsive hydrogel has a lower critical solution temperature (LCST) below about 30° C, below about 29° C, below about 28° C, below about 27° C, or below about 26° C. In related aspects, the thermoresponsive hydrogel has one or more of the following properties: (a) an LCST of about 12° C to 32° C, preferably between about 20° C and 24° C, more preferably of about 22° C; (b) a stiffness of about 100 to 8000 Pascal (Pa), preferably of about 800 to about 1000 Pa; (c) liquid phase viscosity of about 200 to about 4000 cP, preferably of about 200-1000 cP.

[0005] Also provided herein are novel thermoreversible hydrogels useful in the methods described herein.

[0006] Also provided herein is a composition comprising a population of cells produced by the methods described herein, wherein the population of cells is enriched for post-mitotic inhibitory GABAergic cortical interneurons. Preferably, the composition is ECM-free and xeno-free.

[0006a] Also provide herein is a thermoreversible polymer, comprising the formula (III):



wherein (a), (b), (c), and (d) represent mole fractions of co-monomers in the polymer, wherein (a), (b), and (c) are each greater than 0;

PEG_n is a polyethylene glycol polymer and n is an integer;

R^1 , if present, is any terminal or functional group other than a primary amine;

R^2 is a lower alkyl group;

R^3, if present, is a terminal or functional group or linked modifying agent;

R^4 is hydrogen or a lower alkyl group;

L is a linker;

G^1 and G^2 are each independently selected from a polymer segment, a terminal group, a linker and a linked modifying agent,

wherein the average molecular weight of the thermosreversible polymer is greater than 50 kDa, and

wherein the thermoreversible polymer has a weight:weight (w/w) ratio of PEG (component b) to PNIPAAM copolymer backbone (components a, c and d) above 1:2.

[0006b] Also provided herein is a method of preparing a thermoreversible polymer of formula III, the method comprising the steps of (i) polymerizing a comonomer population comprising N-isopropylacrylamide (NIPAAm), alkyl meth/acrylate, and N-acryloxysuccinimide (NASI) in a single solvent in the presence of an initiator concentration effective to form a PNIPAAM-co-PBMA-co-PNASI copolymer backbone; and (ii) reacting the copolymer with a monoPEGamine to form a [PNIPAAM-co-PBMA-co-PNASI]-b-[PEG] copolymer and (iii) reacting the [PNIPAAM-co-PBMA-co-PNASI]-b-[PEG] with isopropylamine to form a thermoreversible polymer of formula III, having a MW of at least 50 kDa.

[0007] Also provided herein is the use of a composition comprising cINs produced by the methods described herein in the treatment of a neurological disorder. In some preferred embodiments, the neurological disorder is epilepsy.

DESCRIPTION OF THE DRAWINGS

[0008] **FIG. 1** is a schematic illustrating two-step synthesis of a thermoreversible poly(NIPAAm-co-Bam)-b-PEG graft copolymer of Formula (III). Mole ratios denoted by lowercase between intermediate reactions. AIBN refers to Azobisisobutyronitrile. Butyl amine is depicted; however, other lower alkyl amines can be substituted in the reaction.

[0009] **FIG. 2** illustrates a comparison of properties of acrylate-based thermoreversible polymers vs. acrylamide-based thermoreversible polymers. LCST = lower critical solution temperature.

[0010] **FIG. 3** illustrates a comparison of properties of low MW acrylate-based thermoreversible polymers vs. high MW acrylate-based thermoreversible polymers. LCST = lower critical solution temperature.

[0011] **FIG. 4** illustrates a comparison of the effects of PEG molecular weight on properties of acrylate-based thermoreversible polymers.

[0012] **FIGS. 5A-D** illustrate a comparison of the effects of PEG:PNIPAAM polymer wt/wt% on properties of acrylate-based thermoreversible polymers. Figs. 5C and 5D show hPSC viability throughout scalable encapsulation processes utilizing different hydrogel formulations. Figure 5C: Varying PEG:PNIPAAm ratios allowed to increase flow rates to meet the minimum required for scale-up of 2mL/min. In addition, the lower shear of the 1:3 formulation maximizes cell viability upon encapsulation (second plot, measured 24hr after encapsulation). Maximal yields are also obtained with the 1:3 formulation. Figure 5D: 3D hydrogel core/shell design,

consisting of a hydrogel+cell core and an outer layer of a-cellular shell. Representative image of encapsulated hPSC aggregates in 3D hydrogel of formula III is shown on the right.

[0013] **FIG. 6** illustrates the effect of isobutyl vs n-butyl at position R^2 of formula III on properties of acrylate-based thermoreversible polymers.

[0014] **FIGS. 6.5A-C** illustrates the effect of -H (hydrogel) vs -CH3 (methyl) at position R^4 of formula III on properties of acrylate-based thermoreversible polymers. 6.5A illustrates the effect on material properties. 6.5B illustrates the differences in temperature based gelation (green = methyl/CH3, grey = hydrogen/H). 6.5C illustrates the effect on hydrogel encapsulation.

[0015] **FIG. 7** illustrates pendant functionalization of the PEG group (R^1 of formula III) with inert structure (methoxy and hydroxyl) and functional structures (acrylate, biotin and DBCO).

[0016] **FIG. 8** illustrates backbone functionalization (R^3 of formula III) of the polymer with methacrylate, maleimide and DBCO.

[0017] **FIG. 9** illustrates protein (FGF and Heparin) presentation and release from the polymer.

[0018] **FIGS. 10A-B** illustrate the effect of various groups (-NH2, -OCH3, and -AC) at the pendant PEG position R^1 of formula III and different solvents on polymer synthesis reaction parameters.

[0019] **FIGS. 11A-E** illustrates a comparison of the generation of MGE progenitor cells from human pluripotent stem cells (hPSCs) using various neural induction protocols, performed in 2-dimensional cell culture and in 3-dimensional thermoreversible hydrogel, as assessed by expression of FOXG1 and DLX1 (qPCR, Figures 11B and 11C) (fold-change relative to undifferentiated hPSCs) and FOXG1 and NKX2-1 (FC, Flow Cytometry, Figures 11D and 11E).

[0020] **FIGS. 12A-F** illustrates a comparison of the generation of cortical interneurons from human pluripotent stem cells (hPSCs) using various neural induction protocols, performed in 2-

dimensional cell culture and in 3-dimensional thermoreversible hydrogel, as assessed by expression of FOXG1, CALB1 and GAD1 (qPCR, Figures 12B-D) (fold-change relative to undifferentiated hPSCs) and FOXG1 and NKX2-1 (FC, Flow Cytometry, Figures 12E-F).

[0021] FIG. 13 Thermoreversible hydrogel for the generation of hPSC-derived cortical interneurons in three-dimensional environments a) Fully defined, synthetic PNIPAM-based hydrogel (is liquid at cold temperatures and quickly gels when heated at 37°C. **b,c).** By controlling the temperature of the system, hPSCs are mixed with the hydrogel at low temperature and extruded into warm culture media leading to the encapsulation of these cells in porous gel beads. These can be used in well plate assays (b) or easily scalable to a perfused bioreactor system (c). Through the use of dedicated media formulations, cells can be differentiated within these beads into any cell type of interest. **d)** Differentiation paradigm and factors used to generate hPSC-derived cINs in 3D hydrogels. **e)** Evolution of marker expression throughout the differentiation process of hPSCs into cINs, and assays used to evaluate differentiation efficacy.

[0022] FIG. 14 Expression of lineage-specific markers of cortical interneuron differentiation. a) On day 10 of differentiation in thermoreversible hydrogel beads, cells begin to express ventral telencephalon marker FoxG1. By day 18, cells express markers of MGE progenitor cells, FoxG1 and NKX2-1. After 35 days of differentiation in hydrogel beads, cells significantly downregulate the expression of NKX2-1, while maintaining high FoxG1 expression, compatible with a mature cIN phenotype. **b)** Representative flow cytometry plots corresponding to the quantification shown in a. Data is represented as mean + s.d. of 2 biological replicates.

[0023] FIG. 15 Gene expression analysis of hPSC differentiation into cortical interneurons. a) Transcript expression analysis by qPCR in cells harvested on day 18 and day 35. Fold change (expressed as $2^{(-\Delta\Delta Ct)}$) relative to gene expression in hPSCs. CALB1 = Calbindin; PV = parvalbumin; SST = somatostatin. **b)** Immunocytochemistry analysis of gene expression on day 35 cells, showing high expression of FoxG1 and NCAM, as well as SST.

Absence of ki67 positive cells indicates post-mitotic cells. **c)** Quantification of b. Data is represented as mean + s.d. of 2 biological replicates.

[0024] FIG. 16. Viability and differentiation efficacy of hPSCs into cortical interneurons encapsulated in thermoreversible hydrogel beads compared to standard two-dimensional processes. a) Post-harvest viability of hPSC-derived cells after 35 days of differentiation in standard 2D cultures or 3D cultures in the novel hydrogel of formula (III) as herein described. **b)** Quantification of a). **c)** Marker expression analyzed by flow cytometry in hPSC-derived cells at different harvest timepoints shows higher differentiation efficacy in the novel hydrogel of formula (III) compared to standard 2D methods (%FoxG1+ cells, fold-change, at days 10, 18 and 35 in 2D vs 3D cultures and %Nkx2-1+ cells, fold change, at day 18). Data is represented as mean + s.d. of 2 biological replicates.

[0025] FIGs. 17A-C illustrates hESC and hiPSC expansion in 3D hydrogels comprising thermoreversible polymers of formula III. Figure 17A: Three culture scales compatible with the 3D hydrogels. Positive-displacement-pipette (PDP) droplets consist of core 3D hydrogel only and allow for rapid screening of multiple culture conditions at small scale. Core/shell beads (C/S beads) – static consist of encapsulated cells in 3D hydrogel beads made of a hydrogel+cell core and an outer layer of a-cellular shell and cultured in static conditions in well plates. C/S beads – Spinner are encapsulated cells in 3D hydrogel core/shell beads that are grown in a spinning environment utilizing spinner flasks. Figure 17B: An example human embryonic stem cell line grown in 3D hydrogel for 8 continuous days achieving fold changes of 80 fold across all 3 culture scales and yields of up to $20e^6$cells/mL 3D hydrogel with high viability upon harvest. Figure 17C: An example of human induced pluripotent stem cell line grown in 3D hydrogel for 8 continuous days achieving fold changes of over 100-fold when grown in encapsulated C/S beads and yields of up to $25e^6$cells/mL 3D hydrogel with high viability upon harvest.

[0026] FIGS. 18A-C Reproducible hPSC expansion in bioreactors at different scales utilizing 3D hydrogels comprising polymers of formula III. Fig. 18A: Schematic of scalable bioreactor system containing 3D hydrogel beads Fig. 18B: Pluripotency marker expression determined by flow cytometry for each lot. FIG. 18C: Gene expression analysis performed by

qPCR, showing strong correlation in gene expression across all 4 lots of hPSC expansion in 3D hydrogel. Pearson's correlation indexes are shown for each plot.

[0027] **FIG. 19. 3D hydrogels comprising polymers of formula III enable efficient hPSC differentiation into pancreatic endoderm cells (PE).** 100-mL Spinner flask contain hPSCs encapsulated in core-shell 3D hydrogel beads. Cells were expanded and differentiated in 3D hydrogel platform. Performance is compared to standard PE differentiation in suspension cultures.

[0028] **FIGS. 20A-D 3D hydrogels comprising polymers of formula III enable efficient hPSC differentiation into pancreatic endoderm cells (PE).** Fig. 20A: Stages of hPSC differentiation into Pancreatic Endoderm (PE) cells. Fig. 20B: Differentiation efficacy was evaluated by expression of the PE marker PDX-1 (pancreatic and duodenal homebox-1). Fig. 20C: Utilizing 3D hydrogel technology allows for a 45-higher production of PE cells compared to standard suspension methods. Fig. 20D: 3D hydrogel technology utilizing improved gel formulation allows for higher control over PE aggregate size compared to standard suspension methods, minimizing the undesired effects of nutrient limitation (such as cell death – necrotic cores – and suboptimal differentiation).

[0029] **FIGS. 21A-C. 3D hydrogels comprising polymers of formula III enable efficient hPSC differentiation into midbrain dopaminergic cells (mDA).** Fig. 21A: 100-mL Spinner flask contain hPSCs encapsulated in core-shell 3D hydrogel beads. Cells were expanded and differentiated in 3D hydrogel platform. Fig. 21B: Upon a 16-day differentiation process utilizing the positive-displacement-pipette (PDP) droplet format, harvested mDA cells presented high viability. Differentiation efficacy was evaluated by the presence of FoxA2, a marker of mDA progenitor cells. Fig. 21C Cells encapsulated in 3D hydrogel C/S beads were expanded and differentiated for 16 days in a 100ml spinner flask format. Harvested mDA cells presented high viability. Differentiation efficacy was evaluated by the presence of FoxA2, a marker of mDA progenitor cells.

[0030] FIGS. 22A-C. 3D hydrogels comprising polymers of formula III enable efficient hPSC differentiation into hematopoietic stem cells (HSCs). Fig. 22A) hPSC differentiation into

HSCs following off-the-shelf media formulation (StemDiff Hematopoietic kit). Fig. 22B) Representative images of hPSCs on day of induction (Stage 1), cultured in hydrogel, and on harvest day (end of Stage 2). Fig 22C) Harvest analytics for hPSC-derived HSCs differentiated in improved hydrogel system.

[0031] FIGS. 23A-C. 3D hydrogels comprising polymers of formula III enable efficient HSC expansion. Fig. 23A) Thaw and expansion of human cord blood-derived CD34+ HSCs in 3D hydrogel. Fig. 23B) Representative images of HSC expanded in hydrogel in SFEM II media (StemDiff Hematopoietic kit) supplemented with StemSpan CD34+ expansion supplement. Fig. 23C) Harvest analytics for HSCs expanded in improved hydrogel system for 8 days.

DETAILED DESCRIPTION OF THE INVENTION

[0032] Definitions

[0032a] Throughout the specification and claims, unless the context requires otherwise, the word “comprise” or variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0033] “Activators,” as used herein, refer to compounds that increase, induce, stimulate, activate, facilitate, or enhance activation the signaling function of the molecule or pathway, e.g., Wnt signaling, SHH signaling, etc.

[0034] As used herein, the term “a population of cells” or “a cell population” refers to a group of at least two cells. In non-limiting examples, a cell population can include at least about 10, at least about 100, at least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, at least about 800, at least about 900, at least about 1000 cells. The population may be a pure population comprising one cell type, such as a population of dopaminergic neurons, or a population of undifferentiated stem cells. Alternatively, the population may comprise more than one cell type, for example a mixed cell population.

[0035] As used herein, the term “stem cell” refers to a cell with the ability to divide for indefinite periods in culture and to give rise to specialized cells.

[0036] As used herein, the term “embryonic stem cell” and “ESC” refer to a primitive (undifferentiated) cell that is derived from preimplantation-stage embryo, capable of dividing

without differentiating for a prolonged period in culture and are known to develop into cells and tissues of the three primary germ layers. A human embryonic stem cell refers to an embryonic stem cell that is from a human embryo. As used herein, the term "human embryonic stem cell" or "hESC" refers to a type of pluripotent stem cells derived from early-stage human embryos, up to and including the blastocyst stage, that is capable of dividing without differentiating for a prolonged period in culture, and are known to develop into cells and tissues of the three primary germ layers.

[0037] As used herein, the term "embryonic stem cell line" refers to a population of embryonic stem cells which have been cultured under *in vitro* conditions that allow proliferation without differentiation for up to days, months to years.

[0038] As used herein, the term "pluripotent" refers to an ability to develop into the three developmental germ layers of the organism including endoderm, mesoderm, and ectoderm.

[0039] As used herein, the term "induced pluripotent stem cell" or "iPSC" refers to a type of pluripotent stem cell formed by the introduction of certain embryonic genes (such as but not limited to OCT4, SOX2, and KLF4 transgenes) (see, for example, Takahashi and Yamanaka Cell 126, 663-676 (2006), herein incorporated by reference) into a somatic cell.

[0040] As used herein, the term "neuron" refers to a nerve cell, the principal functional units of the nervous system. A neuron consists of a cell body and its processes—an axon and one or more dendrites. Neurons transmit information to other neurons or cells by releasing neurotransmitters at synapses.

[0041] As used herein, the term "undifferentiated" refers to a cell that has not yet developed into a specialized cell type.

[0042] As used herein, the term "differentiation" refers to a process whereby an unspecialized embryonic cell acquires the features of a specialized cell such as a neuron, heart, liver, or muscle cell. Differentiation is controlled by the interaction of a cell's genes with the physical and

chemical conditions outside the cell, usually through signaling pathways involving proteins embedded in the cell surface.

[0043] As used herein, the term "inducing differentiation" in reference to a cell refers to changing the default cell type (genotype and/or phenotype) to a non-default cell type (genotype and/or phenotype). Thus, "inducing differentiation in a stem cell" refers to inducing the stem cell (e.g., human stem cell) to divide into progeny cells with characteristics that are different from the stem cell, such as genotype (e.g., change in gene expression as determined by genetic analysis such as a microarray) and/or phenotype (e.g., change in expression of one or more protein markers).

[0044] As used herein, the term "marker" or "cell marker" or "biomarker" refers to gene or protein that identifies a particular cell or cell type. A marker for a cell may not be limited to one marker, markers may refer to a "pattern" of markers such that a designated group of markers may identity a cell or cell type from another cell or cell type.

[0045] As used herein, the term "linker" or "linkage" refers to a linking moiety that connects two groups and has a backbone of 100 atoms or less in length. A linker or linkage may be a covalent bond that connects two groups or a chain of between 1 and 100 atoms in length, for example of 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18 or 20 carbon atoms in length, where the linker may be linear, branched, cyclic or a single atom. In certain cases, one, two, three, four or five or more carbon atoms of a linker backbone may be optionally substituted with a sulfur, nitrogen or oxygen heteroatom. The bonds between backbone atoms may be saturated or unsaturated, usually not more than one, two, or three unsaturated bonds will be present in a linker backbone. The linker may include one or more substituent groups, for example with an alkyl, aryl or alkenyl group. A linker may include, without limitations, poly(ethylene glycol); ethers, thioethers, tertiary amines, alkyls, which may be straight or branched, e.g., methyl, ethyl, n-propyl, 1-methylethyl (isopropyl), n-butyl, n-pentyl, 1,1-dimethylethyl (t-butyl), and the like. The linker backbone may include a cyclic group, for example, an aryl, a heterocycle or a cycloalkyl group, where 2 or more atoms, e.g., 2, 3 or 4 atoms, of the cyclic group are included in the backbone. A linker may be cleavable or non-cleavable.

[0046] "Alkyl" refers to monovalent saturated aliphatic hydrocarbyl groups having from 1 to 10 carbon atoms and such as 1 to 6 carbon atoms, or 1 to 5, or 1 to 4, or 1 to 3 carbon atoms. In some cases, a "lower alkyl" is an alkyl group having 1 to 6 carbon atoms. This term includes, by way of example, linear and branched hydrocarbyl groups such as methyl (CH_3—), ethyl (CH_3CH_2—), n-propyl ($CH_3CH_2CH_2$—), isopropyl ($(CH_3)_2CH$—), n-butyl ($CH_3CH_2CH_2CH_2$—), isobutyl ($(CH_3)_2CHCH_2$—), sec-butyl ($(CH_3)(CH_3CH_2)CH$—), t-butyl ($(CH_3)_3C$—), n-pentyl ($CH_3CH_2CH_2CH_2CH_2$—), and neopentyl ($(CH_3)_3CCH_2$—).

[0047] The term "substituted alkyl" refers to an alkyl group as defined herein wherein one or more carbon atoms in the alkyl chain have been optionally replaced with a heteroatom such as —O—, —N—, —S—, —$S(O)_n$— (where n is 0 to 2), —NR— (where R is hydrogen or alkyl) and having from 1 to 5 substituents selected from the group consisting of alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, acyl, acylamino, acyloxy, amino, aminoacyl, aminoacyloxy, oxyaminoacyl, azido, cyano, halogen, hydroxyl, oxo, thioketo, carboxyl, carboxylalkyl, thioaryloxy, thioheteroaryloxy, thioheterocyclooxy, thiol, thioalkoxy, substituted thioalkoxy, aryl, aryloxy, heteroaryl, heteroaryloxy, heterocyclyl, heterocyclooxy, hydroxyamino, alkoxyamino, nitro, —SO-alkyl, —SO-aryl, —SO-heteroaryl, —SO_2-alkyl, —SO_2-aryl, —SO_2-heteroaryl, and —NR^aR^b, wherein R and R may be the same or different and are chosen from hydrogen, optionally substituted alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, heteroaryl and heterocyclic.

[0048] As used herein, the terms "chemoselective functional group" and "chemoselective tag" are used interchangeably and refer to chemoselective reactive groups that selectively react with one another to form a covalent bond. Chemoselective functional groups of interest include, but are not limited to, two thiol groups, thiols and maleimide or iodoacetamide, as well as groups that can react with one another via Click chemistry, e.g., azide and alkyne groups (e.g., cyclooctyne groups). Chemoselective functional groups of interest, include, but are not limited to, thiols, alkyne, a cyclooctyne, an azide, a phosphine, a maleimide, an alkoxyamine, an aldehyde and protected versions thereof, and precursors thereof. In certain embodiments, the chemoselective functional group is a thiol.

[0049] In some embodiments, the term "terminal group" refers to group(s) which are produced as a result of any convenient method of polymerization of the subject co-monomers described herein, e.g., H, an alkyl or a substituted alkyl and/or a residual component of an initiator used during polymerization.

[0050] As used here, the term "modifying agent" refers to any convenient agent that provides for a desirable property of interest (e.g., a desirable physical and/or biological property) and which is capable of conjugation to the thermoreversible polymer, e.g., via a chemoselective functional group on a sidechain linker or terminal of the polymer. Such an agent may belong to the class of small molecule, protein, peptide, sugar, polynucleotide, etc. Modifying agents of interest include, but are not limited to, a ligand, a substrate, an enzyme, a pharmaceutical agent (e.g., a chemotherapeutic agent), a plasmid, a polynucleotide, a bioactive peptide, an antibody, a biomarker, a bio-sensor, a catalyst, an element, a cell targeting agent, small drug molecules, fluorescent/radioactive/optical imaging agents, peptides/proteins/enzymes, nucleic acids (siRNA/RNA/DNA/etc.), metal based compounds/catalysts, site-specific cellular targeting agents (compounds/ligands/antibodies/etc.) and smart adjuvants, gene therapy vectors. In certain embodiments, the modifying agent is selected from a heparin, a hyaluronic acid, a specific binding member, a peptide, a nucleic acid, gelatin, fibronectin, collagen, laminin, bFGF, EGF, insulin, progesterone, glucose, thymosin beta-4, SHH, Noggin, Activin, TGFb3, FGF8, BDNF, GDNF, NT3, PDGF-AA and IGF-1. In certain instances, the modifying agent is a cytokine, a BMP family member (e.g., TGFbeta or activin), a neutrophin (e.g., NT3 or BDNF) or a hedgehog protein (e.g., SHH).

[0051] Any convenient methods may be utilized in conjugating a modifying agent to a thermoreversible polymer. Conjugation methods and chemistries of interest include, but are not limited to, those described by Greg Hermanson in Bioconjugate Techniques (Third edition) 2013, Academic Press. In certain embodiments, the modifying agent is a protein. In certain embodiments, the modifying agent is a peptide. In certain embodiments, the modifying agent is peptidic and can be conjugated to the thermoreversible polymer (e.g., via a terminal and/or a sidechain functional group) by covalent attachment to the N-terminal or C-terminal or the peptidic agent, or covalent attachment to an amino acid sidechain (e.g., an amino, thiol,

hydroxyl, carboxylic acid or phenol-containing amino acid sidechain group, or a derivative thereof). In certain embodiments, the modifying agent is a heparin. In certain embodiments, the heparin modifying agent is linked via a thiol linkage. In certain instances, the heparin can be linked to the subject polymers via conjugation to a carboxylic acid group of the heparin. For example, FIG. 9 depicts exemplary methods of linking thiol-heparin to an acrylate group of the polymer by Michael addition. In certain embodiments, two or more modifying agents (e.g., a heparin and a hyaluronic acid) may be linked to each other in addition to a thermoreversible polymer.

[0052] As used, herein the lower critical solution temperature (LCST) refers to the critical temperature below which the components of a mixture are miscible for all compositions. The word lower in the term indicates that the LCST is a lower bound to a temperature interval of partial miscibility, or miscibility for certain compositions only.

[0053] The present inventors have discovered that the methods described herein enable increases in production of MGE progenitor cells of at least 10-fold compared to standard 2-dimensional culture formats, with at least 4-fold higher cell viability upon harvest.

[0054] In some aspects, an *in vitro* method for differentiating human stem cells is provided, the method comprising: encapsulating human stem cells in a three dimensional synthetic hydrogel and contacting the encapsulated human stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling and with at least one Wingless (Wnt) antagonist for a predetermined amount of time; and contacting the cells with at least one activator of Sonic Hedgehog (SHH) signaling for a predetermined amount of time to obtain a cell population comprising MGE progenitor cells. In some aspects, the cell population comprising MGE progenitor cells exhibits the following features: (a) at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 86% or at least 87% of the cell population is FoxG1-positive; and (b) at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, or at least 77% of the cell population is NKX2-1-positive. In some aspects, FOXG1-positive NKX2-1-positive MGE progenitor cells comprise at least at least 60%, at least 65%, at least 70%, at least 75%, or at least 80% of the cell population, e.g., as measured by flow cytometry, immunocytochemistry and/or qPCR.

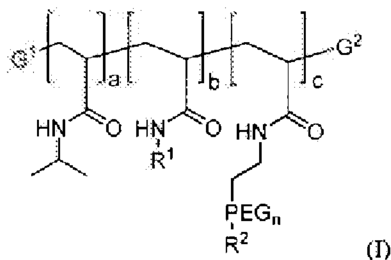
[0055] In preferred aspects, the method further comprises contacting the produced encapsulated MGE progenitor cells with at least one neurotropic factor and optionally a notch inhibitor for a predetermined period of time to obtain a population of cells comprising FOXG1-positive differentiated inhibitory GABAergic cortical interneurons (cINs). In some aspects, the cell population comprising cINs exhibits the following features: (a) at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, or at least 77% of the cell population is FoxG1-positive; (b) at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, or at least 84% of the cell population is GABA-positive; (c) at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, or at least 83% of the cell population is PV-positive; (d) less than 30%, less than 25%, less than 24%, or less than 23% of the cell population is NKX2-1-positive and/or (e) less than 5%, less than 4%, less than 3%, less than 2% or less than 1% of the cell population is ki67-positive, as measured by e.g., flow cytometry, immunocytochemistry and/or qPCR.

[0056] Synthetic Hydrogels

[0057] In some embodiments, the synthetic hydrogel for use in the methods described herein is a polyethylene glycol-poly(N-isopropylacrylamide) (PEG-PNIPAAM) based hydrogel that is solid at 37° C. Preferred PEG-PNIPAAM hydrogels include those described in U.S. Patent No. 10,982,055, and WIPO publication No. 2022/251137A1, the entire contents of each of which are incorporated herein by reference.

[0058] In some preferred embodiments, the hydrogel comprises a thermoreversible polymer comprising: a N-isopropylacrylamide (NIPAAM) co-monomer; a lower alkyl amine co-monomer; and a poly(ethylene glycol) (PEG) co-monomer, wherein the terminal PEG monomer is substituted with alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, cycloalkyl, substituted cycloalkyl, heterocycloalkyl, substituted heterocycloalkyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, heteroaryl, substituted heteroaryl, heteroarylalkyl, and substituted heteroarylalkyl. Preferably, the lower alkyl amine co-monomer comprises n-butyl, isobutyl, tert-butyl, n-propyl, pentyl, isopropyl, or isopentyl; and the terminal PEG monomer is substituted with an alkoxy group.

[0059] In some embodiments, the thermoreversible polymer comprises the formula (I):



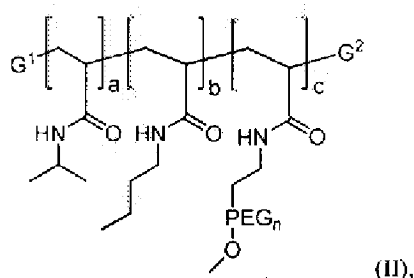
(I)

wherein: a, b, and c are molar fractions of the co-monomers, where a, b, and c are each greater than zero, preferably $a > 0.8$; $0.2 > b > 0$; and $0.1 > c > 0$; PEG_n is a polyethyleneglycol polymer and n is an integer from 1 to 2500;

R^1 is an alkyl or a substituted alkyl, preferably C1-C6 alkyl, more preferably butyl;

R^2 is alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, cycloalkyl, substituted cycloalkyl, heterocycloalkyl, substituted heterocycloalkyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, heteroaryl, substituted heteroaryl, heteroarylalkyl, and substituted heteroarylalkyl, preferably is alkoxy, more preferably methoxy; and G^1 and G^2 are each independently selected from a polymer segment, a terminal group, a linker and a linked modifying agent.

[0060] In related embodiments, the thermoreversible polymer comprises the formula (II):



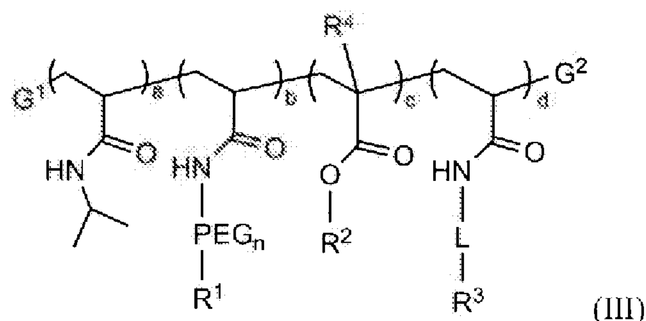
(II),

wherein n is 1 to 2500; and

G^1 and G^2 are each independently selected from a polymer segment, a terminal group, a linker and a linked modifying agent.

[0061] Other synthetic hydrogels may be utilized in the method, including, without limitation, those described in U.S. Patent Nos. 6,897,064 and 10,982,055 and U.S. Patent Publication No. 2024/0294713 the entire contents of each of which are incorporated herein by reference.

[0062] Also provided herein are novel thermoreversible polymers comprising the formula III, as well as 3-dimensional hydrogels comprising the novel thermoreversible polymers and their use in the methods described herein (e.g., culturing and/or differentiating stem cells):



(III)

wherein a, b, c, d represent molar fractions of the co-monomers in the polymer, wherein a, b, and c are each greater than 0.
PEG_n is a polyethylene glycol polymer
R^1, if present, is any terminal or functional group not including a primary amine
R^2 is a lower alkyl group
R^3, if present, is a terminal or functional group or linked modifying agent
R^4 is hydrogen or a lower alkyl group
G^1 and G^2 are each independently selected from a polymer segment, a terminal group, a linker and a linked modifying agent,

and wherein the molecular weight of the polymer is greater than 50 kDa.

[0063] The present inventors have discovered that thermoreversible polymers of formula III have several advantages over the thermoreversible polymers disclosed e.g., in U.S. Patent No. 10,982,055 and U.S. Patent Publication No. 2024/0294713. Briefly, as exemplified herein, a thermoreversible polymer according to the present disclosure exhibits a combination of properties rendering them uniquely suitable for 3-dimensional cell culture. By way of example, a 3-dimensional hydrogel comprising a thermoreversible polymer of formula III exhibits, *inter alia*, (1) long-term stability, enabling lengthy differentiation processes to occur during cell

culture (2) decreased viscosity relative to prior art polymers, enabling efficient cell encapsulation and (3) appropriate stiffness supporting cell growth. These features yield significant improvements in cell viability and cell yield.

[0064] In preferred aspects, the thermoreversible polymer of formula III has one or more, preferably all, of the following properties: (a) LCST of 12-32° C (b) stiffness of 100-8000 Pa (c) viscosity of 100-2000 cP and (d) molecular weight of 50-500 kDa.

[0065] In some aspects, R^1 of the thermoreversible polymer of formula III is absent. In other aspects, R^1 of the thermoreversible polymer of formula III is a functional group. In some aspects, the functional group is a chemoselective functional group, nonlimiting examples of which include two thiol groups, thiols and maleimide or iodoacetamide, as well as groups that can react with one another via Click chemistry, e.g., azide and alkyne groups (e.g. cyloalkyne groups such as dibenzocyclooctyne (DBCO)). Functional groups include, but are not limited to, acrylate, thiols, hydroxyl, alkoxy (e.g., methoxy), alkyne, cycloalkyne, an azide, hydrazide, a phosphine, a maleimide, a carboxylic acid, an alkoxyamine, an aldehyde, biotin, silane, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), NHS ester, toluenesulfonyl (Tos), and protected versions and precursors thereof.

[0066] In some aspects, R^1 of the thermoreversible polymer of formula III is a terminal group, nonlimiting examples of which include, without limitation, C_1-C_6 alkoxy (e.g., methoxy, ethoxy, n-propoxy, n-butoxy isobutoxy, tert-butoxy, pentoxy or isopentoxy), alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, cycloalkyl, substituted cycloalkyl, heterocycloalkyl, substituted heterocycloalkyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, heteroaryl, substituted heteroaryl, heteroarylalkyl, and substituted heteroarylalkyl. In some aspects, R^1 is other than an alkyl (e.g., is other than n-butyl) or substituted alkyl.

[0067] In some aspects, R^2 of the thermoreversible polymer of formula III is a lower alkyl, optionally selected from methyl, ethyl, propyl, n-butyl, pentyl, isopropyl, isobutyl, isopentyl, tert-butyl, cyclopropyl, and cyclobutyl. In some preferred embodiments, R^2 is n-butyl. The present inventors have discovered that n-butyl at this position exhibits higher stiffness with

similar viscosity, higher gel stability and lower gel LCST compared to other conformations such as iso-butyl. In some embodiments, R^2 is a lower alkyl other than isobutyl.

[0068] In some aspects, R^3 of the thermoreversible polymer of formula III is absent. In other aspects, R^3 of the thermoreversible polymer of formula III is a modifying agent, optionally selected from selected from a heparin, a hyaluronic acid, a specific binding member, a peptide, a nucleic acid, gelatin, fibronectin, collagen, laminin, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin, progesterone, glucose, stromal cell derived factor-1 (SDF-1), thymosin beta-4, sonic hedgehog (SHH), Noggin, Activin, transforming growth factor-β (TGF-β), FGF8, brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), neurotrophic factor-3 (NT3), platelet-derived growth factor (PDGF), IL-16, IL-2 and insulin-like growth factor-1 (IGF-1).

[0069] In some preferred embodiments, R^4 is a lower alkyl group, particularly where R^4 is methyl.

[0070] In some instances G^1 and G^2 are each independently selected from a polymer segment, a terminal group, a linker and a linked modifying agent. In related aspects, G^1 and G^2 are each independently selected from a heparin, a hyaluronic acid, a member of a specific binding pair, a polypeptide, a nucleic acid and a carboxyl group. In related aspects, G^1 and G^2 are each independently a modifying agent selected from gelatin, elastin, fibronectin, collagen, and laminin. In related aspects, G^1 and G^2 are each independently selected from a chemokine, a peptide hormone, and a growth factor. In some embodiments, G^1 and G^2 are each independently selected from fibroblast growth factor, epidermal growth factor, hepatic growth factor insulin, stromal cell-derived factor- 1, thymosin beta-4, sonic hedgehog, Noggin, activin, transforming growth factor, bone morphogenic protein, brain- derived neurotrophic factor, glial cell-derived neurotrophic factor, neurotrophin-3, platelet-derived growth factor, FGF-2, FGF-8, keratinocyte growth factor, or insulin-like growth factor. In some aspects, G^1 and G^2 are each independently selected from a chain-transfer agent non-limiting examples of which include a dithioester, dithiocarbamate, trithiocarbonate or a xanthate. In some aspects, G^1 and G^2 are each

independently selected from a chain transfer agent containing a thiolcarbonylthio group and a thermal initiator such as Azobisisobutyronitrile (AIBN).

[0071] In some instances, $a > 0.8$; $0.1 > b > 0$; $0.2 > c > 0$, and $0.1 > d > 0$.

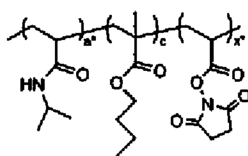
[0072] In some aspects, a thermoreversible polymer of formula III has a molecular weight (MW) of between 50-500 kDa or between 50-250 kDa. In certain embodiments, the thermoreversible polymer has a MW of at least 50 kDa, at least 60 kDa, at least 70 kDa, at least 80 kDa or at least 90 kDa. In certain embodiments, the thermoreversible polymer has a MW in the range of about 50 kDa to about 250 kDa, such as about 50 kDa to about 200 kDa, about 50 kDa to about 150 kDa, about 50 kDa to about 100 kDa, or about 50 kDa to about 75 kDa. In certain embodiments, the thermoreversible polymer has a MW of about 60 kDa to about 140 kDa, about 70 kDa to about 130 kDa, about 80 kDa to about 120 kDa or about 90 kDa to about 110 kDa. In some embodiments, the MW is about 80 kDa, about 85 kDa, about 90 kDa, about 95 kDa, about 100 kDa, about 105 kDa, about 110 kDa, about 115 kDa, or about 120 kDa. In certain preferred embodiments, the thermoreversible polymer has a MW of about 50 kDa to about 250 kDa or from about 50 to about 150 kDa.

[0073] Any convenient poly(ethylglycol) (PEG) polymeric groups may be utilized as a sidechain in the thermoreversible polymers of Formula (III). In some embodiments of formula (III), PEG_n is a polyethylglycol polymer having a MW of about 2-20 kDa. In related embodiments, PEG_n has a MW of about 2 kDa or greater, such as 2 kDa to 20 kDa, or 2 kDa to 10 kDa, or 3 kDa to 20 kDa, or 3 kDa to 10 kDa. In some embodiments, the PEG_n has a MW of about 2 kDa to about 9 kDa, about 3 kDa to about 8 kDa, about 4 kDa to about 7 kDa, about 4 kDa to about 6 kDa. In some embodiments, the PEG_n has a MW of about 2 kDa, about 3 kDa, about 4 kDa, about 5 kDa, about 6 kDa, about 7 kDa, about 8 kDa or about 9 kDa. The PEG_n group can be modified with any convenient groups, including terminal modifications..

[0074] In certain embodiments, a thermoreversible polymer of formula III has a weight:weight (w/w) ratio of PEG to PNIPAAm copolymer above 1:2. In some embodiments the w/w ratio of PEG to PNIPAAm copolymer is about 1:2.5, about 1:2.75, about 1:3.0, about 1:3.5, about 1:3.75, about 1:4.0, about 1:4.25, or about 1:4.5. In some embodiments, the w/w

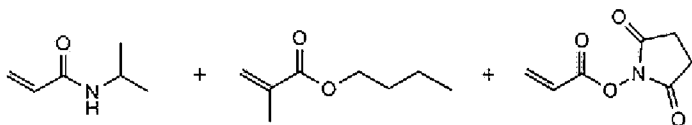
ratio of PEG to PNIPAAm copolymer is about 1:3 to about 1:4.5, or about 1:4. In some embodiments, the w/w ratio of PEG to PNIPAAm copolymer is measured according to the relative molecular ratio with nuclear magnetic resonance (NMR) and the total polymer molecular weight with gel permeation chromatography (GPC) (GPC-NMR analysis).

[0075] In some aspects, the weight of the PNIPAAm copolymer is defined as the weight of the copolymer backbone (i.e., prior to addition of PEG) following polymerization of the co-monomers. For example, in some aspects, the weight of the PNIPAAm copolymer is defined as the weight of:

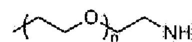


[PNIPAAm-*co*-PBMA-*co*-PNASI]

following polymerization of the comonomers:



N-isopropylacrylamide   Butyl methacrylate   **N-acryloxysuccinimide**



methoxyPEGamine

In related aspects, the weight of PEG is defined as the weight of [methoxyPEGamine] prior to reaction with the copolymer backbone.

[0076] In particularly preferred embodiments, a thermoreversible polymer of formula II comprises a PEG:PNIPAAM weight ratio above 1:2 and a MW between 50 kDa and 250 kDa (including any range therebetween).

[0077] The present inventors have discovered that a polymer of formula III having PEG:PNIPAAM weight ratios above 1:2 (e.g., ratio of 1:3) and low MW (e.g., 50kDa-250 kDa) provides several surprising advantages over polymers disclosed in, e.g., U.S. Patent No. 10,982,055 and U.S. Patent Publication No. 2024/0294713, including, but not limited to, (1) hydrogels with lower viscosity, minimizing shear during the cell encapsulation process for large scale bioreactor cell culture systems (2) faster flow rates, reaching 2mL/min and (3) higher control of bead size and geometry (higher circularity and lower frequency of tail formation) (4) maintenance of three-dimensional structure during lengthy cell differentiation processes and optimal stiffness range to support expansion and differentiation of stem cells into all three germ layers and (5) improved functionalization ability for controlled protein presentation and release. A thermoreversible polymer of formula III therefore provides a scalable three-dimensional cell culture system over time periods relevant for e.g., production of functional neurons.

[0078] In certain embodiments, a thermoreversible polymer of formula III has an LCST in the range of 12-32° C, such as 12-30° C., 15-30° C., 15-25° C. or 10-20° C. In preferred embodiments, the thermoreversible polymer of formula III has an LCST of about 20° C to about 22° C.

[0079] Also provided is a composition comprising: a) a 3-dimensional hydrogel comprising a thermoreversible polymer of Formula III; and b) cells encapsulated within the hydrogel. The 3-dimensional hydrogel-cell composition is useful for generating a desired number of cells, by culturing the 3-dimensional hydrogel-cell composition under conditions and for a period of time sufficient to generate the desired number of cells. In some aspects, the period of time sufficient to generate the desired number of cells is at least 14 days, at least 15 days, at least 16 days, at least 17 days, at least 18 days, at least 19 days, at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 40 days, at least 45 days, at least 50 days, at least 55 days, at least 60 days, at least 3 months, at least 4 months, at least 6 months, or more. Such cells can include stem cells, differentiated cells, and the like. A thermoreversible polymer-cell composition of Formula III is particularly useful for differentiating cells, e.g., to generate a

desired number of differentiated cells over a relatively long period of time. A 3-dimensional thermoreversible polymer-cell composition of the present disclosure can be implanted into an individual in need thereof, where cells proliferate and/or differentiated within the implanted thermoreversible polymer-cell composition, and migrate out of the implanted thermoreversible polymer-cell composition.

[0080] In some aspects, the present disclosure provides a composition comprising a plurality of hydrogel capsules, wherein at least 90%, at least 95%, at least 98% or at least 99% of said hydrogel capsules in said composition comprise a cell and a hydrogel encapsulating said cell, wherein said hydrogel encapsulating said cell comprises a thermoreversible polymer of formula III. In some embodiments, at least 90% of said hydrogel capsules each comprises multiple cells, e.g., at least 100, at least 200, at least 500, at least 700, at least 800, at least 900 or more cells. As shown in the working examples of the present disclosure, a thermoreversible polymer of formula III provides improvements, including but not limited to, bead (i.e., capsule) homogeneity, bead circularity, bead sphericity, reduction in bead volume and diameter, flow rate to create the beads, and shape of the beads from gravity drip extrusion.

[0081] The present disclosure provides methods of producing differentiated cells from a stem cell or a precursor cell, the methods comprising culturing a stem cell or precursor cell in a 3-dimensional hydrogel composition comprising a thermoreversible polymer of Formula III, for a period of time and under conditions suitable for inducing differentiation of the stem cell or precursor cell. Conditions for inducing differentiation of a stem cell or precursor cell depend in part on the desired differentiated cell. Conditions can include inclusion in the hydrogel of one or more factors that induce differentiation. In some aspects, the conditions suitable for differentiation of the stem cells comprise culturing the stem cell or precursor cell in the hydrogel composition for a period of at least 14 days, at least 15 days, at least 16 days, at least 17 days, at least 18 days, at least 19 days, at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 40 days, at least 45 days, at least 50 days, at least 55 days, at least 60 days, at

least 3 months, at least 4 months, at least 6 months, or more to produce the differentiated cell population.

[0082] In several aspects, the present disclosure provides methods for expanding stem cells, the method comprising encapsulating single cells or multicellular clusters in a three-dimensional hydrogel comprising a thermoreversible polymer of formula III and culturing the cells under suitable stem cell expansion conditions.

[0083] The subject thermoreversible polymers can be prepared using any convenient methods. A variety of polymerization methods may be utilized in preparing a base polymeric material, e.g., including polyacrylate, polyacrylamide and mixtures thereof. A variety of derivatization methods may be utilized to introduce any convenient functionality into the subject base polymeric materials. A variety of chemoselective conjugation chemistries, linkers, functional groups and modifying agents may be utilized in the preparation of further derivatives and conjugates of the subject base polymeric materials and derivatives thereof.

[0084] In some aspects, a method of preparing a thermoreversible polymer of formula III is provided, comprising the steps of (i) polymerizing a comonomer population comprising N-isopropylacrylamide (NIPAAm), alkyl methacrylate (preferably butyl methacrylate (BMA)) and N-acryloxysuccinimide (NASI) in a single ideal solvent that maximizes monomer solubility to form a PNIPAAM-co-PBMA-co-PNASI copolymer backbone and (ii) reacting the copolymer with a monoPEGamine (e.g., methoxyPEGamine) to form a [PNIPAAM-co-PBMA-co-PNASI]-b-[PEG] copolymer and (iii) reacting the [PNIPAAM-co-PBMA-co-PNASI]-b-[PEG] with isopropylamine to form a thermoreversible polymer of formula III. Optimally, the concentration of initiator in step (i) controls the backbone molecular weight and thus the total polymer molecular weight. In preferred aspects, the w/w ratio of the PEGamine to the PNIPAAM-co-PBMA-co-PNASI copolymer backbone (controlled by initiator concentration) to the is greater than 1:2 and is preferably above 1:3. In some embodiments the w/w ratio of the PEGamine to the PNIPAAM-co-PBMA-co-PNASI copolymer is about 1:2.5, about 1:2.75, about 1:3.0, about 1:3.5, about 1:3.75, about 1:4.0, about 1:4.25, or about 1:4.5. In some embodiments, the w/w ratio of PEGamine to PNIPAAm copolymer is about 1:3 to about 1:4.5, or about 1:4. In some embodiments, a thermoreversible polymer of formula III is provided produced by such a process.

[0085] In some aspect, the single solvent is selected from Acetone, Acetonitrile, Benzene, Chloroform, Dichloromethane, Dimethylformamide, Dimethyl sulfoxide, Dioxane, Ethyl acetate, Pyridine, Ethanol, Methanol, Tetrahydrofuran, Toluene, and Water.

[0086] Stem Cells

[0087] The term "stem cell" refers to a cell that is totipotent or pluripotent or multipotent and is capable of differentiating into one or more different cell types. The term includes without limitation as embryonic stem cells, stem cells isolated from organs, for example, skin stem cells, and induced pluripotent stem cells (iPSC). The term "totipotent" refers to an ability of a cell to differentiate into any type of cell in a differentiated organism, as well as cell of extraembryonic materials such as placenta. As used herein, the term "induced pluripotent stem cell" or "iPSC" refers to a type of pluripotent stem cell that is similar to an embryonic stem cell but is created when somatic (e.g., adult) cells are reprogrammed to enter an embryonic stem cell-like state by being forced to express factors important for maintaining the "stemness" of embryonic stem cells (ESCs), i.e., their ability to be led to commit to different differentiation pathways. As used herein, the term "progenitor" in reference to a cell refers to an intermediate cell stage wherein said cell is no longer a pluripotent stem cell and is also not yet a fully committed cell. Progenitor cells in this disclosure are included within somatic cells.

[0088] The term "pluripotent" refers to a cell line capable of differentiating into any terminally differentiated cell type.

[0089] The term "multipotent" refers to a cell line capable of differentiating into at least two terminally differentiated cell types.

[0090] The term "embryonic stem cell" refers to a primitive (undifferentiated) cell that is derived from preimplantation-stage or early-stage embryo (for example, up to and including the blastocyst stage), capable of dividing without differentiating for a prolonged period in culture, and capable of developing into cells and tissues of the three primary germ layers. Embryonic stem cells can also be isolated from an embryo or placenta or umbilical cord.

[0091] The term "embryonic stem cell line" refers to a population of embryonic stem cells which have been cultured under *in vitro* conditions that allow proliferation without differentiation for days, months or even years (e.g. human embryonic stem cell lines SA01, VUB01, HUES 24, H1, H9, WT3, HUES1).

[0092] The term "induced pluripotent stem cell" or "iPSC" refers to a type of pluripotent stem cell, similar to an embryonic stem cell, formed by the introduction of certain embryonic genes (such as a OCT4, SOX2, and KLF4 trans genes) (see, for example, Takahashi and Yamanaka Cell 126, 663-676 (2006), herein incorporated by reference) into a somatic cell. Examples of somatic cells include, but are not limited to, bone marrow cells, epithelial cells, fibroblast cells, hematopoietic cells, hepatic cells, intestinal cells, mesenchymal cells, myeloid precursor cells and spleen cells. Alternatively, the iPSC can be produced by reprogramming a somatic cell to enter an embryonic stem cell-like state by being forced to express factors important for maintaining the "stemness" of embryonic stem cells (ESCs), i.e., their ability to be led to commit to different differentiation pathways.

[0093] Methods for culturing stems cells, particularly human embryonic stem cells, are known in the art and described in WO2006/029297, WO2006/019366 and WO2006/029198 all to Thomson and Ludwig, and WO2008/089351 to Bergendahl and Thomson, which are hereby incorporated by reference in their entirety.

[0094] <u>Inhibitors of "Small Mothers Against Decapentaplegic" (SMAD)</u>

[0095] Small Mothers against Decapentaplegic (SMAD) refers, generally, to a class of signaling molecules that are capable of modulating the directed cell differentiation of stem cells. SMADs are intracellular proteins that transduce extracellular signals from transforming growth factor beta ligands to the nucleus where they activate downstream gene transcription and are members of a class of signaling molecules capable of modulating directed differentiation of stem cells.

[0096] In reference to the presently disclosed methods, inhibitors of SMAD signaling include compounds that interact with and reduce or block the activity of SMAD and/or a molecule that is

associated with SMAD or other component molecules of SMAD signaling. Inhibitors can bind directly to and cause a conformational change in SMAD signaling, can reduce or prevent the expression of the gene encoding SMAD or a SMAD target gene, can decrease SMAD protein levels, and/or can interfere with SMAD interactions with one or more signaling partners.

[0097] Inhibitors also include molecules that indirectly regulate SMAD biological activity by intercepting upstream signaling molecules (e.g., within the extracellular domain), examples of a signaling molecule and an effect include: Noggin which sequesters bone morphogenic proteins, inhibiting activation of ALK receptors 1, 2, 3, and 6, thus preventing downstream SMAD activation. Likewise, Chordin, Cerberus, Follistatin, similarly sequester extracellular activators of SMAD signaling. Bambi, a transmembrane protein, also acts as a pseudo-receptor to sequester extracellular TGFβ signaling molecules.

[0098] Antibodies that block activins, nodal, TGFβ, and BMPs are contemplated for use to neutralize extracellular activators of SMAD signaling, and the like). Thus in one embodiment, an inhibitor of the present disclosure induces (changes) or alters differentiation from a default to a non-default cell type, for example, one of the methods of the present disclosure comprising at least three inhibitors that produced a non-default neural progenitor cell.

[0099] Inhibitors disclosed herein "alter" or "lower" or "block" default signaling in order to direct cellular differentiation towards a nondefault cell type, such as described herein for producing cortical interneurons as disclosed herein. Thus, an inhibitor of the present disclosure can be a biological compound, natural or synthetic, or a small molecule for increased or decreased signal molecule activity that assists in producing for example cortical interneurons of the present disclosure.

[0100] Inhibitors are described in terms of competitive inhibition (binds to the active site in a manner as to exclude or reduce the binding of another known binding compound) and allosteric inhibition (binds to a protein in a manner to change the protein conformation in a manner which interferes with binding of a compound to that protein's active site) in addition to inhibition induced by binding to and affecting a molecule upstream from the named signaling molecule that in turn causes inhibition of the named molecule.

[0101] SMAD inhibitors that may be advantageously employed in the methods disclosed herein include those SMAD inhibitors that are well known in and are readily available to those having skill in the art. SMAD inhibitors that have been employed for the neural conversion of human ESCs and iPSCs are described in Chambers et al., *Nat Biotechnol* 27:275-280 (2009).

[0102] Exemplary SMAD inhibitors that can be used in the methods and compositions disclosed herein include the compounds designated SB431542, LDN-193189, Noggin PD169316, SB203580, LY364947, A77-01, A-83-01, BMP4, GW788388, GW6604, SB-505124, lerdelimumab, metelimumab, GC-I008, AP-12009, AP-11014, LY550410, LY580276, LY364947, LY2109761, SB-505124, E-616452 (RepSox ALK inhibitor), SD-208, SMI6, NPC-30345, Ki26894, SB-203580, SD-093, activin-M108A, P144, soluble TBR2-Fc, DMH-1, Dorsomorphin dihydrochloride, and a derivative and/or variant thereof, wherein each derivative and/or variant thereof possesses one or more SMAD inhibitory activities.

[0103] *a) TGFβ/Activin/Nodal Pathway Inhibitors*

[0104] Inhibition of SMAD signaling pathway includes inhibition of TGFβ/Activin/Nodal pathway and the BMP pathway. Exemplary TGFβ/activin pathway inhibitors include but are not limited to: TGF beta receptor inhibitors, inhibitors of SMAD 2/3 phosphorylation, inhibitors of the interaction of SMAD 2/3 and SMAD 4, and activators/agonists of SMAD 6 and SMAD 7. Furthermore, the categorizations described below are merely for organizational purposes and one of skill in the art would know that compounds can affect one or more points within a pathway, and thus compounds may function in more than one of the defined categories.

[0105] TGF beta receptor (e.g., ALK5) inhibitors can include antibodies to, dominant negative variants of, and antisense nucleic acids that suppress expression of, TGF beta receptors (e.g., ALK5). Exemplary TGFβ receptor/ALK5 inhibitors include, but are not limited to, SB431542 (see, e.g., Inman, et al., Molecular Pharmacology 62(1):65-74 (2002)), A-83-01, also known as 3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide (see, e.g., Tojo, et al., Cancer Science 96(11):791-800 (2005), and commercially available from, e.g., Toicris Bioscience); 2-(3-(6-Methylpyridin-2-yl)-IH-pyrazol-4-yl)-1,5-naphthyridine, Wnt3a/BIO (see, e.g., Dalton, et at., WO2008/094597, herein incorporated by reference), BMP4

(see, Dalton, supra), GW788388 (-{4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl}-N(tetrahydro-2H-pyran-4-yl)benzamide) (see, e.g., Gellibert, et al., Journal of Medicinal Chemistry 49(7):2210-2221 (2006)), SM16 (see, e.g., Suzuki, et al., Cancer Research 67(5):2351-2359 (2007)), IN-I 130 (3-((5-(6-methylpyridin-2-yl)-4-(quinoxalin-6-yl)-IHimidazol-2-yl)methyl)benzamide) (see, e.g., Kim et al., *Xenobiotica* 38(3):325-339 (2008)), GW6604 (2-phenyl-4-(3-pyridin-2-yl-IH-pyrazol-4-yl)pyridine) (see, e.g., de Gouville, et al., Drug News Perspective 19(2):85-90 (2006)), SB-505124 (2-(5-benzo[1,3]dioxol-5-yl-2-tertbutyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride) (see, e.g., DaCosta et al., *Molecular Pharmacology* 65(3):744-752 (2004)) and pyrimidine derivatives (see, e.g., those listed in WO2008/006583, herein incorporated by reference). In some aspects, the method comprises the use of the SMAD inhibitor SB431542 at a concentration of about 5-15 μM, preferably about 10 μM for a period of about 5 days to about 10 days, preferably about 7 to 8 days.

[0106] Further, while "an ALK5 inhibitor" is not intended to encompass non-specific kinase inhibitors, an "ALK5 10 inhibitor" should be understood to encompass inhibitors that inhibit ALK4 and/or ALK7 in addition to ALK5, such as, for example, SB-431542. See, e.g., Inman et al., *J Mol Phamacol* 62(1):65-74 (2002). Without intending to limit the scope of the disclosure, it is believed that ALK5 inhibitors affect the mesenchymal to epithelial conversion/transition (MET) process. TGFβ/activin pathway is a driver for epithelial to mesenchymal transition (EMT). Therefore, inhibiting the TGFβ/activin pathway can facilitate MET (i.e., reprogramming) process.

[0107] Specific examples of inhibitors include but are not limited to SU5416; 2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride (SB-505124); lerdelimumb (CAT-152); metelimumab (CAT-192); GC-I008; ID11; AP-12009; AP-11OI4; LY550410; LY580276; LY364947; LY2109761; SB-505124; SB-431542; SD-208; SMI6; NPC-30345; Ki26894; SB-203580; SD-093; Gleevec; 3,5,7,2′,4′-entahydroxyflavone (Morin); activin-M108A; P144; soluble TBR2-Fc; and antisense transfected tumor cells that target TGF beta receptors. See, e.g., Wrzesinski et al., *Clinical Cancer Research* 13(18):5262-

5270 (2007); Kaminska et al., *Acta Biochimica Polonica* 52(2):329-337 (2005); and Chang et al., *Frontiers in Bioscience* 12:4393-4401 (2007).

[0108] Inhibitors of SMAD 2/3 phosphorylation can include antibodies to, dominant 5 negative variants of and antisense nucleic acids that target SMAD2 or SMAD3. Specific examples of inhibitors include PD169316; SB203580; SB-431542; LY364947; A77-01; and 3,5,7,2',4'-pentahydroxyflavone (Morin). (See, e.g., Wrzesinski, supra; Kaminska, supra; Shimanuki, et al., Oncogene 26:3311-3320 (2007); and Kataoka, et at., EP 1992360, incorporated herein by reference.)

[0109] SB-431542 (i.e., CAS 301836-41-9; IUPAC 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]-benzamide) is a commercially available small molecule inhibitor of SMAD, which is capable of lowering or blocking transforming growth factor beta (TGFβ)/Activin-Nodal signaling.

[0110] Inhibitors of the interaction of SMAD 2/3 and SMAD 4 can include antibodies to, dominant negative variants of and antisense nucleic acids that target SMAD2, SMAD3 and/or smad4. Specific examples of inhibitors of the interaction of SMAD 2/3 and SMAD4 include but are not limited to Trx-SARA, Trx-xFoxH1b and Trx-Lef1. (See, e.g., Cui, et al., Oncogene 24:3864-3874 (2005) and Zhao, et al., Molecular Biology of the Cell, 17:3819-15 3831 (2006).)

[0111] *(b) BMP Inhibitors*

[0112] Exemplary BMP pathway inhibitors include, but are not limited to: Noggin, BMP receptor inhibitors, inhibitors of SMAD 1/5/8 phosphorylation, inhibitors of the interaction of SMAD 1/5/8 and SMAD 4, and activators/agonists of SMAD 6 and SMAD 7. The categorizations described below are merely for organizational purposes and one of skill in the art would know that compounds can affect one or more points within a pathway, and thus compounds may function in more than one of the defined categories.

[0113] Inhibitors of SMAD 1/5/8 phosphorylation include, but are not limited to, antibodies to, dominant negative variants, antisense nucleic acids, and small molecules that target SMAD 1,

SMAD 5, or SMAD 8. Specific examples of inhibitors include LDN-193189 and Dorsomorphin (commercially available from, e.g., Stemgent)

[0114] BMP receptor inhibitors include, but are not limited to, antibodies to, dominant negative variants of, siRNA or antisense nucleic acids, or small molecules that target BMP receptors. Specific examples of inhibitors include, but are not limited to, DMH-1, Dorsomorphin dihydrochloride, and LDN-193189 (commercially available, from, e.g., Tocris Biosciences).

[0115] LDN193189 (i.e., DM-3189, IUPAC 4-(6-(4-(piperazin-1-yl)phenyl)pyrazolo[1,5-a]pyrimidin-3-yl)quinolone) is a commercially available small molecule inhibitor of SMAD signaling. LDN193189 is also a highly potent small molecule inhibitor of ALK2, ALK3, ALK6, protein tyrosine kinases (PTK), inhibiting signaling of members of the ALK1 and ALK3 families of type I TGFβ receptors, resulting in the inhibition of the transmission of multiple biological signals, including the bone morphogenetic proteins (BMP) BMP2, BMP4, BMP6, BMP7, and Activin cytokine signals and subsequently SMAD phosphorylation of Smad1, Smad5, and Smad8. Yu et al., *Nat Med* 14:1363-1369 (2008) and Ctmy et al., *Bioorg Med Chem Lett* 18: 4388-4392 (2008). In some aspects, the method comprises the use of the BMP inhibitor LDN-193189 at a concentration of about 50-150 nM, preferably about 100 nM for a period of about 10 days to about 20 days, preferably about 14-16 days.

[0116] (c) *Dual SMAD Inhibitors*

[0117] SMAD signaling inhibitors can comprise the dual-SMAD inhibitors SB431542 and LDN-193189, or functional derivatives and/or variants thereof. As used herein, the terms "LSB" and "XLSB" have been described previously in the Definitions section. Dual SMAD inhibitors substantially increase the efficiency of the differentiation.

[0118] According to the methods of the present disclosure, SB431542 can be contacted with the pluripotent and/or a multipotent cell at a final concentration in an *in vitro* culture of from about 0.1 μM to about 1 mM. LDN-193189 can be contacted with the pluripotent and/or a multipotent cell at a final concentration in an *in vitro* culture of from about 1 nM to about 10 μM.

[0119] Antagonists of Wingless (Wnt) Signaling

[0120] "Wingless" or "Wnt" refers to a signal pathway composed of Wnt family ligands and Wnt family receptors, such as Frizzled and LRPDerailed/RYK receptors, mediated with or without β-catenin. Wnt proteins have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis.

[0121] The Wnt pathway includes any of the proteins downstream or upstream of Wnt protein activity. For example, this could include LRPS, LRP6, Dkk, GSK-3, Wnt10B, Wnt6, Wnt3 (e.g., Wnt 3A), Wntl or any of the other proteins discussed herein, and the genes that encode these proteins.

[0122] The Wnt pathway also includes pathways that are downstream of Wnt, such as the LRPS or HBM pathways, the Dkk pathway, the p-catenin pathway, the MAPKAPK2 pathway, the OPG/RANK pathway, and the like. By "LRP5 pathway" and "IBM pathway" is meant any proteins/genes including LRP5 or the HBM mutant and proteins downstream of LRPS or the HBM mutant. By "β-catenin pathway" is meant any proteins/genes including β-catenin and proteins downstream of β-catenin. By "MAPKAPK2 pathway" is meant any proteins/genes including MAPKAPK2 and proteins downstream of MAPKAPK2. By "OPG/RANKL pathway" is meant any proteins/genes including OPG/RANKL and proteins downstream of OPG and RANKL. By "Dkk pathway" is meant to include any proteins/genes involved in Dkk-1 and LRP5 and/or LRP6 interaction that is part of the Wnt pathway. Dkk-I inhibits LRP5 activity.

[0123] The term "Wnt antagonist" as used herein refers not only to any agent that may act by directly inhibiting the normal function of the Wnt protein, but also to any agent that inhibits the Wnt signaling pathway, and thus recapitulates the function of Wnt. Examples of the Wnt signaling antagonists include XAV939 (Hauang et al. *Nature* 461:614-620 (2009)), vitamin A (retinoic acid), lithium, flavonoid, Dickkopfl (Dkk1), insulin-like growth factor-binding protein (IGFBP) (WO2009/131166), and siRNAs against β-catenin.
Exemplary Wnt antagonists include, but are not limited to, XAV939, IWP-2, DKK1 (Dickkopf protein 1), and IWR1. Additional Wnt inhibitors include, but are not limited to, IWR compounds, IWP compounds, and other Wnt inhibitors described in WO09155001 and Chen et

al., *Nat Chem Biol* 5:100-7 (2009). In some aspects, the method comprises the use of the Wnt antagonist IWP-2 at a concentration of about 1-10 μM, preferably about 5 μM for a period of about 5 days to about 10 days, preferably about 7 or 8 days.

[0124] XAV939 is a potent, small molecule inhibitor of tankyrase (TNKS) 1 and 2 with IC_{50} values of 11 and 4 nM, respectively. Huang et al., *Nature* 461:614-620 (2009). By inhibiting TNKS activity, XAV939 increases the protein levels of the axin-GSK3β complex and promotes the degradation of β-catenin in SW480 cells. Known antagonists of Wnt signaling also include Dickkopf proteins, secreted Frizzled-related proteins (sFRP), Wnt Inhibitory Factor 1 (WIF-1), and Soggy. Members of the Dickkopf-related protein family (Dkk-1 to -4) are secreted proteins with two cysteine-rich domains, separated by a linker region. Dkk-3 and -4 also have one prokineticin domain. Dkk-1, -2, -3, and -4 function as antagonists of canonical Wnt signaling by binding to LRP5/6, preventing LRP5/6 interaction with Wnt-Frizzled complexes. Dkk-1, -2, -3, and -4 also bind cell surface Kremen-1 or -2 and promote the internalization of LRP5/6. Antagonistic activity of Dkk-3 has not been demonstrated. Dkk proteins have distinct patterns of expression in adult and embryonic tissues and have a wide range of effects on tissue development and morphogenesis.

[0125] The Dkk family also includes Soggy, which is homologous to Dkk-3 but not to the other family members. The sFRPs are a family of five Wnt-binding glycoproteins that resemble the membrane-bound Frizzleds. The largest family of Wnt inhibitors, they contain two groups, the first consisting of sFRP1, 2, and 5, and the second including sFRP3 and 4. All are secreted and derived from unique genes, none are alternate splice forms of the Frizzled family. Each sFRP contains an N-terminal cysteine-rich domain (CRO). Other antagonists of Wnt signaling include WIF-1 (Wnt Inhibitory Factor 1), a secreted protein that binds to Wnt proteins and inhibits their activity.

[0126] In some embodiments, the present disclosure relates to inhibitors and/or antagonists of the SMAD and Wnt signaling pathways. SMAD inhibitors include, but not limited to, SB431542, LDN-193189, Noggin PD169316, SB203580, LY364947, A77-01, A-83-01, BMP4, GW788388, GW6604, SB-505124, lerdelimumb, metelimumab, GC-I008, AP-12009, AP-110I4,

LY550410, LY580276, LY364947, LY2109761, SB-505124, SB-431542, SD-208, SMI6, NPC-30345, Ki26894, SB-203580, SD-093, activin-M108A, P144, soluble TBR2-Fc, DMH-1, Dorsomorphin dihydrochloride and their derivatives. Wnt antagonists include, but not limited to, XAV939, DKK1, SFRP-1, SFRP-2, SFRP-5, SFRP-3, SFRP-4, WIF-1, Soggy, IWP-2, IWR1 and their derivatives.

[0127] In some aspects of these methods, SB431542 and LDN193189 can be used in combination to inhibit SMAD signaling pathway. In other aspects, XAV939 can be employed to antagonize the Wnt signaling pathway.

[0128] In other aspects of these methods, the concentration of XAV939 in a cell culture can be about 0.2 μM to 20 μM; the concentration of LDN193189 in a cell culture can be from about 10 nM to about 1000 nM, and the concentration of SB431542 in a cell culture can be from about 1 μM to about 100 μM. For example, the concentrations of XAV939 can be about 2 μM, the concentration of LDN193189 can be about 100 nM, and the concentration of SB431542 can be about 10 μM.

[0129] In further aspects of these methods, forebrain progenitor cells are generated by contacting stem cells with XAV939, LDN193189, and/or SB431542 for a duration of from about 5 days to about 40 days. In related aspects, forebrain progenitor cells are generated by contacting stem cells with XAV939, LDN193189, and/or SB431542 for a duration of from about 10 days to about 25 days.

[0130] Within further aspects of the methods according to any of these three embodiments, Wnt signaling antagonists can be selected from the group consisting of XAV939, DKK1, DKK-2, DKK-3, Dkk-4, SFRP-1, SFRP-2, SFRP-5, SFRP-3, SFRP-4, WIF-1, Soggy, IWP-2, IWR1, ICG-001, KY0211, Wnt-059, LGK974, IWP-L6, and a derivative and/or variant thereof, wherein each derivative and/or variant thereof possesses one or more Wnt signaling antagonist activities. For example, the Wnt signaling antagonist can comprise XAV939 or a functional derivative and/or variant thereof. XAV939 can be contacted with the pluripotent and/or multipotent cell at a final concentration in an *in vitro* culture of from about 10 nM to about 500 μM.

[0131] A variety of cell culture media and supplement can be used to differentiate stem cells into forebrain progenitor cells, including KSR-medium, N2 medium (DMEM/F12 with NaHCO3, N2B supplement (Stem Cell Technologies), and Neurobasal media with B27 (Gibco) and N2 supplements (Invitrogen). Kriks et al., *Nature* 480:547-551 (2011). In some embodiments, the cells are maintained on mouse embryonic fibroblasts (MEFs) as described previously and dissociated with Accutase (Innovative Cell Technologies) for differentiation or dispase for passaging (Chambers et al., *Nat Biotechnol* 27:275-280 (2009)).

[0132] Activators of Sonic Hedgehog (SHH)

[0133] The ventral prosencephalic progenitor population transcription factor marker, NKX2.1, can be used to monitor differentiation. Sussel et al., *Development* 126:3359-3370 (1999) and Xu et al., *J Neurosci* 24:2612-2622 (2004). Inhibition of Wnt signaling enhances the production of FOXG1 and, subsequently, promotes ventralization by inducing the controlled, SHH-mediated differentiation of multipotent and pluripotent cells towards an NKX2.1+ forebrain progenitor fate.

[0134] The generation of such neuronal cell lineages and populations may be achieved by contacting a neuronal precursor cells, such as a neuronal precursor cell generated as disclosed herein, with one or more activator of SHH signaling at a predetermined time after contacting a multipotent and/or pluripotent cell with one or more inhibitors of SMAD signaling and one or more antagonists of Wnt signaling and for a duration that is sufficient to induce the production of one or more markers of a cortical interneuron or precursor thereof.

[0135] As used herein, the term "activator" in the context of SHH refers to a compound that promotes and/or enhances SHH signaling thereby inducing a neuronal precursor cell to differentiate into a cortical interneuron or precursor thereof, a hypothalamic neuron or precursor thereof, and/or a pre-optic chorionic neuron or precursors thereof. Examples of the SHH signaling pathway activators useful in the present disclosure include proteins belonging to the hedgehog family (e.g., SHH), inhibitors of Ptc's interaction with Smo, Smo receptor activators, Shh receptor activators (e.g., Hg—Ag, purmorphamine, etc.), substances increasing Ci/Gli

family levels, inhibitors of the intracellular degradation of Ci/Gli factors, and SHH overexpression constructs or Ci/Gli overexpression constructs resulting from transfection.

[0136] In some aspects of the present methods, an SHH signaling pathway activator, (e.g., SHH+ purmorphamine), is added to the cell culture for the full or partial duration of the culture. In some embodiments, the concentrations of SHH activators in cell culture are about 10 ng/mL to about 5000 ng/mL for SHH (or recombinant SHH) and about 0.1 μM to about 20 μM for purmorphamine. In some preferred embodiments, the concentrations of SHH activators in cell culture are about 50 ng/mL to about 500 ng/mL for SHH (or recombinant SHH) and about 0.5 μM to about 4 μM for purmorphamine.

[0137] Purmorphamine is a commercially available small molecule with the name 9-cyclohexyl-N-[4-(morpholinyl)phenyl]-2-(1-naphthalenyloxy)-9H-purin-6-amine, and a chemical formula of $C_{31}H_{32}N_6O_2$. The structure of purmorphamine listed below. Purmorphamine binds to and activates the 7-transmembrane Smo receptor of the Hedgehog signaling pathway.

[0138] Within still further aspects of the methods according to any of these three embodiments, SHH signaling activators can be selected from the group consisting of Smoothened agonist (SAG), SAG analog, SHH, C25-SHH, C24-SHH, purmorphamine, Hg-Ag and a derivative and/or variant thereof, wherein each derivative and/or variant thereof possesses one or more SMAD inhibitory activities. For example, the SHH signaling activator can comprise recombinant SHH and purmorphamine, or functional derivatives and/or variants thereof. Recombinant SHH can be contacted with the pluripotent and/or multipotent cell at a final concentration in an *in vitro* culture of from about 5 ng/mL to about 5 μg/mL. Purmorphamine can be contacted with the pluripotent and/or multipotent cell at a final concentration in an *in vitro* culture of from about 0.1 μM to about 20 μM. In some aspects, the method comprises the use of the SSH activator SAG at a concentration of about 0.05-5 μM, preferably about 0.1 μM for a period of about 15 days to about 25 days, preferably about 21 or 22 days.

[0139] The difference in timing of SHH differentiation activation is crucial to trigger the generation of distinct ventral progenitors of divergent anterior-posterior identity. Early activation of SHH signaling in the derivation of hESC-derived progenitors expressing markers of the

hypothalamic anlage requires the presence of FGF-8. Kriks et al., *Nature* 480:547-551 (2011). Neuronal precursor cells can be contacted with the one or more activator of SHH signaling after the passage of a predetermined period of time following generation of the neuronal precursor cell and/or following the contacting of the pluripotent and/or multipotent cell with the one or more inhibitors of SMAD and/or the one or more antagonists of Wnt signaling.

[0140] For example, contacting the neuronal precursor cell with one or more activator of SHH signaling can be initiated from about 4 days to about 20 days or from about 8 days to about 18 days after contacting a pluripotent cell and/or a multipotent cell with one or more inhibitors of SMAD and with one or more antagonists of Wnt signaling. Contacting the neuronal precursor cell with one or more activator of SHH signaling can be for a time period of from about 5 days to about 30 days or from about 8 days to about 16 days.

[0141] An "FGF receptor (FGFR) agonist" as used herein means a molecule that can activate FGFR (e.g. molecules that bind to FGFR and induce the dimerization of the receptor and activate the signaling P13K pathway and Ras/ERK pathway). Nonlimiting examples of FGFR agonists include FGF2, FGF8 and SUN11602. In preferred embodiments, the FGFR agonist is FGF8 (e.g. recombinantly produced FGF8). According to the methods described herein, the cells are contacted with an FGFR agonist such as FGF8 in order to shift the balance towards rostralization. In some aspects, the cells are contacted with FGF8 for a period of about 10 to about 20 days, preferably for a period of about 12 days to about 16 days, more preferably for about 13, 14, 15 or 16 days. Preferably, contact of the cells with an FGFR agonist such as FGF8 is initiated about 5 to about 10 days, preferably about 6 days, 7 days or 8 days, after initial contact of the cells with one or more SMAD inhibitors and/or after initial contact of the cells with a WNT inhibitor and/or after initial contact of the cells with an SHH activator.

[0142] In some preferred aspects, the method comprises (i) LDN193189 (LDN) to inhibit BMP signaling (ii) SB-431542 (SB) to inhibit TGFβ signaling (iii) recombinant FGF8 (iv) smoothened agonist (SAG; 3-chloro-N-[trans-4-(methylamino)cyclohexyl]-N-[3-(pyridin-4-yl)benzyl]-1-benzothiophene-2-carboxamide) to activate sonic hedgehog signaling (iv) IWP2 to inhibit WNT signaling and (v) FGF8 to shift the balance towards rostralization.

[0143] Further Maturation

[0144] MGE progenitor cells may be differentiated into cINs, preferably post-mitotic cINs, according to the methods described herein by contacting the cells with neurotrophic factors such as, without limitation, glial cell line derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) for a predetermined amount of time. Preferably, the cells are simultaneously contacted with a notch inhibitor such as DAPT. In some aspects, the predetermined amount of time is at least 10 days, at least 11 days, at least 12 days, at least 13 days, at least 14, days, at least 15 days, at least 16 days, at least 17 days, at least 18 days, at least 19 days, at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, or at least 30 days.

[0145] Markers of Cortical Interneurons

[0146] As used herein, the term "marker" or "cell marker" refers to gene or protein that identifies a particular cell or cell type. A marker for a cell may not be limited to one marker. Markers may refer to a "pattern" of markers such that a designated group of markers may identify a cell or cell type from another cell or cell type.

[0147] Markers of cortical interneurons and/or of cortical interneuron precursor cells have been described and are readily available to those of skill in the art and include, for example, SST, PV, GABA, calbindin, LHX6, RAX, FOXA2, FOXG1, OLIG2, MASH1, NKX6.2, VGLUT1, MAP2, CTIP2, SATB2, TBR1, DLX2, ASCL1, and ChAT.

[0148] In some aspects, differentiation into a post-mitotic cIN precursor cell is identified by expression of one or more markers selected from FOXG1, PV, SST, Calbindin, DCX, ASCL1, TUJ1, GABA, GAD1, VGAT, VGLUT1, and GAD67. In related aspects, a post-mitotic cIN precursor cell is identified by lack of expression of one or more makers selected from NKX2-1 and OLIG2 and optionally Ki67.

[0149] In some aspects, differentiation into a telencephalon cell is identified by expression of FoxG1 and/or lack of expression of RAX.

[0150] In some aspects, differentiation into a ventral telencephalon cell is identified by expression of FoxG1 and DLX2 and/or lack of expression of EMX1.

[0151] In some aspects, differentiation into an MGE progenitor cell is identified by expression of one or more markers selected from FOXG1, NKX2-1, NKX2-2, ASCL1, SIX6, OLIG2, NKX6.2, DLX1/2 and LHX6.

[0152] In related aspects, a post-mitotic cIN precursor cell is distinguished from an MGE progenitor cell by detecting expression of at least NKX2-1 and OLIG2, wherein an MGE progenitor cell is identified by expression of NKX2-1 and lack of expression of OLIG2 and a post-mitotic cIN precursor cell is identified by expression of OLIG2 and lack of expression of NKX2-1.

[0153] It will be understood that pluripotent cells and/or multipotent cells can be human cells or murine cells, which can be selected from the group consisting of embryonic stem cells, adult stem cells, neural stem cells, induced pluripotent cells, engineered pluripotent cells, primary progenitor cells, induced progenitor cells, and engineered progenitor cells.

[0154] Contacting with SMAD inhibitors and/or contacting with Wnt signaling antagonists can be carried out simultaneously or can be carried out sequentially. Contacting can be for a duration of from about 5 days to about 30 days.

[0155] Methods by the current disclosure can be used to produce cortical interneurons and their precursors, at an amount and purity that cannot be obtained by the state of the art. A large number of pure, functional cortical interneurons obtained by using methods of the present disclosure, in some embodiments, can be used to study seizures, schizophrenia or autism, and other neurological diseases. Similarly, these cells can also be used in cell therapy.

[0156] Compositions

[0157] Within further embodiments, the present disclosure provides compositions comprising one or more *in vitro* differentiated neuronal cells that produce one or more markers of a cortical interneuronal cell and/or of a cortical interneuron precursor cell, wherein the *in vitro* differentiated neuronal cells are produced by: (a) contacting a multipotent cell or a pluripotent cell encapsulated in a synthetic hydrogel with two or more inhibitors of SMAD signaling, (b) contacting the multipotent cell or a pluripotent cell with one or more inhibitors of Wnt signaling, and (c) contacting the multipotent cell or a pluripotent cell with one or more activators of SHH signaling.

[0158] Composition disclosed herein can comprise a mixture of two or more cells wherein cortical interneurons comprise at least about 30% of the total number of cells, or at least about 40% of the total number of cells, or at least about 50% of the total number of cells, or at least about 60% of the total number of cells, or at least about 70% of the total number of cells, or at least about 80% of the total number of cells, or at least about 90% of the total number of cells, or at least about 95% of the total number of cells.

[0159] Compositions can comprise a mixture of two or more cells wherein NKX2.1+/PV+ cortical interneurons comprise at least about 5%, or at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, at least about 50%, at least about 60%, at least about 70% or at least about 80% of the total number of cells in the composition.

[0160] Compositions can comprise a mixture of two or more cells wherein γ-aminobutyric acid (GABA)-ergic inhibitory interneurons comprise at least about 5%, or at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, at least about 50%, at least about 60%, at least about 70% or at least about 80% of the total number of cortical interneurons in the composition.

[0161] Cortical interneuron or interneuron precursor cells can be modified with a transgene expressing a detectable marker such as, for example, CT-2 or green fluorescence protein (GFP). It will be understood that these detectable markers can be interchanged with other detectable markers without deviating from this aspect of the present disclosure.

[0162] The cortical interneuron precursor cells of the present invention give rise to functional interneurons, exhibiting the morphological, neurochemical, and electrophysiological properties of mature interneurons. The immature interneuron precursor preparation can mature *in vitro* under controlled culture conditions mimicking their *in vivo* neuronal environment. Alternatively, the immature interneuron precursor cells mature following transplantation and migration within the cerebral cortex of a mammalian subject (e.g., a human subject). The immature interneuron precursor cells of the present invention can migrate extensively, in a non-radial (i.e., tangential) fashion upon transplantation into the cerebral cortex. Upon migration, the cortical interneuron precursor cells can mature into parvalbumin and Kv3.1 expressing interneurons that exhibit fast spiking action potential discharge patterns. Alternatively, the cortical interneuron precursor cells of the present invention can mature into somatostatin expressing interneurons, exhibiting the characteristic rebound, adapting, non-fast spiking firing patterns of this sub-group of interneurons. These somatostatin expressing interneurons may further express neuropeptide Y.

[0163] The cortical interneuron precursor cells can mature into interneurons having an average resting membrane potential of about −40 mV to about −70 mV. Overtime, the average resting membrane potential becomes more hyperpolarized, ranging from about −55 mV to about −70 mV.

[0164] Methods for the Treatment of Disorders and Disease

[0165] *In vitro* derivation of neuronal cells from stem cells or progenitor cells have significant clinical implications and are also important for disease modeling and drug screening. Conditions suitable for treatment in accordance with this method of the present invention include, without limitation seizure disorders, such as epilepsy or infantile spasms; neuropsychiatric disorders, such as autism, schizophrenia, an anxiety disorder, and an eating disorder; neurodevelopmental disorders, such as holoprosencephaly or microcephaly; and Parkinson's disease. Early studies using hPSCs have been primarily geared towards neurodegenerative disorders, which are known to affect specific neuron types such as midbrain dopamine neurons in Parkinson's disease (PD; (Kriks et al., *Nature* 480:547-551 (2011); Soldner et al., *Cell* 136:964-977 (2009); and Soldner et al., *Cell* 146:318-331 (2011)) or motor neurons in amyotrophic lateral sclerosis (ALS; Dimos et al., *Science* 321:1218-1221 (2008)) and spinal muscular atrophy (SMA; Ebert et

al., *Nature* 457:277-280 (2009)). More recent studies suggest the possibility of tackling complex neuronal disorders such as Schizophrenia (Brennand et al., *Nature* 473:221-225 (2011)) or autism-related syndromes (Marchetto et al., *Cell* 143:527-539 (2010) and Pasca et al., *Nat Med* 17:1657-1662 (2011)).

[0166] The cells of the present disclosure can be delivered via intraparenchymal or intraventricular transplantation as described in U.S. Pat. Nos. 5,082,670 and 5,650,148 to Gage et al., and U.S. Patent Publication No. 20060141622 to Johe et al., which are hereby incorporated by reference in their entirety. Intraparenchymal transplantation can be achieved by injecting the immature interneuron precursor cells within the host brain parenchyma or by preparing a cavity by surgical means to expose the host brain parenchyma and then depositing the cell graft into the cavity. Both methods provide parenchymal apposition between the grafted cells and host brain tissue at the time of grafting, and both facilitate anatomical integration between the graft and host brain tissue. Alternatively, the graft may be placed in a ventricle, e.g., a cerebral ventricle or subdurally, e.g., on the surface of the host brain where it is separated from the host brain parenchyma by the intervening pia mater or arachnoid and pia mater. Grafting to the ventricle may be accomplished by injection of the donor cells or by growing the cells in a substrate such as 30% collagen to form a plug of solid tissue which may then be implanted into the ventricle to prevent dislocation of the graft. For subdural grafting, the cells may be injected around the surface of the brain after making a slit in the dura. This is of importance if it is required that the graft become an integral part of the host brain and to survive for the life of the host.

[0167] Regardless of the survival issue, transplantations of neuronal cells and precursors described by the present disclosure result in the successful grafting of large numbers of cells that can be studied after their maturation *in vivo*.

[0168] Methods for the Treatment of Neurodegenerative Disorders and Disease

[0169] In certain embodiments, the present disclosure provides methods for the treatment of disorders and diseases that are associated with neurodegeneration including, for example, seizure disorders, Parkinson's disease (PD) and Alzheimer's disease (AD), which methods comprise the

in vivo administration of cortical interneurons to a patient afflicted with a seizure disorder, PD or AD, which cortical interneurons are generated by the methods disclosed herein.

[0170] Methods for the Treatment of Psychiatric Disorders and Disease

[0171] In other embodiments, the present disclosure provides methods for the treatment of psychiatric disorders and diseases including, for example, schizophrenia and the autism-related disorders.

[0172] Unlike in PD, ALS, or SMA, the neuron types critical for modeling schizophrenia or autism are less well defined, and no attempts have been made to direct neuron subtype identity in those studies. Great strides have been made recently in establishing protocols for the derivation of human ESC-derived cortical projection neurons. Espuny-Camacho et al., *Neuron* 77:440-456 (2013) and Shi et al., *Nat Neurosci* 15:477-486, S471 (2012).

[0173] However, inhibitory neurons, such as cortical interneurons may have a particularly important role in schizophrenia or autism. Insel, *Nature* 468:187-193 (2010) and Lewis et al., *Nat Rev Neurosci* 6:312-324 (2005).

[0174] Current paradigms for the modeling and treatment of human psychiatric disease employ patient-specific iPSC-derived neurons. Brennand et al., *Nature* 473:221-225 (2011); Cheung et al., *Hum Mol Genet* 20:2103-2115 (2011); Chiang et al., *Mol Psychiatry* 16:358-360 (2011); Marchetto et al., *Cell* 143:527-539 (2010); and Pasca et al., *Nat Med* 17:1657-1662 (2011). Those published studies were, however, performed in mixed neural cultures of unclear neuronal subtype identity and with limited characterization of subtype specific synaptic and functional properties. A convergence of post-mortem findings has been exploited in an attempt to link genetic defects to psychiatric disorders, such as the interneuron-associated Erbb4 receptor in schizophrenia. Fazzari et al., *Nature* 464:1376-1380 (2010).

[0175] As disclosed herein, the present disclosure provides purified populations of mature cortical interneurons that may be used as models for human psychiatric disorders and diseases and in therapeutic regimen for the treatment of such psychiatric disorders and diseases.

Moreover, the data presented herein demonstrate that highly efficient derivation of cortical interneurons is possible following timed exposure to developmental cues.

[0176] While not wishing to be bound by theory, it is believed that putative hESC-derived GABAergic interneurons receive synaptic inputs from other human interneurons and from excitatory mouse projection neurons. Cells exhibiting the neurochemical properties of cortical interneurons adopt fairly mature physiological properties within 30 days of plating on mouse cortex cultures. While the mechanisms of accelerated *in vitro* maturation of the NKX2.1:GFP+ neurons on mouse cortical cultures are currently unknown, the involvement of species-specific timing factors derive from the data presented herein. The presently disclosed data further demonstrate that synaptically active cortical interneurons can be derived *in vitro* and can be useful for modeling and treatment of cortical interneuron pathologies in psychiatric disorders including, but not limited to, schizophrenia and autism.

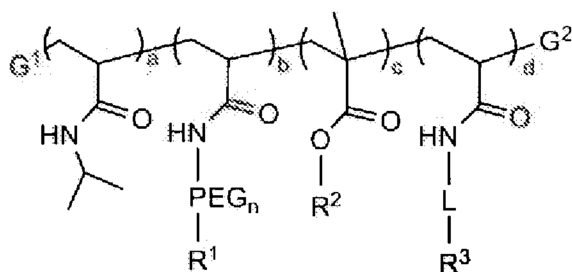
[0177] The generation of hESC-derived PV expressing neurons and the presence of relatively rapid spiking, non-accommodating neurons in these cultures is of particular interest given the implications of PV interneuron dysfunction in schizophrenia. Beasley and Reynolds *Schizophr Res* 24:349-355 (1997) and Woo et al., *Am J Psychiatry* 154:1013-1015 (1997). Fast-spiking PV+ cortical interneurons are observed late during primate prenatal development and continue their maturation into early adulthood. Anderson et al., *Neuroscience* 67:7-22 (1995) and Insel, *Nature* 468:187-193 (2010). Given the role of PV+ neurons under various pathological conditions, the data presented herein support the modeling of such dysfunctional neuronal states and the treatment of those dysfunctional neuronal states by the administration of cortical interneurons and/or precursors thereof.

[0178] Thus, within certain aspects of these embodiments, the present disclosure provides the generation of enriched cortical interneuron subgroups, such as somatostatin+ and PV+ cells. MGE progenitors may be under control of SHH signaling, with high SHH signaling levels promoting the generation of somatostatin+ cells and lower SHH signaling levels promoting the generation of PV+ neurons. Xu et al., *Neuron* 65:328-340 (2010).

[0179] Any methods known in the art for measuring gene expression may be used, in particular, quantitative methods such as, real time quantitative PCR or microarrays, or methods using gene reporter expression or qualitative methods such as immunostaining or cell sorting methods identifying cells exhibiting specific biomarkers, including cell surface markers.

[0180] Several embodiments of the invention are exemplified in the following Items:

[0181] Item 1. A thermoreversible polymer with improved stability over time, comprising the formula (III):



wherein a, b, c, d represent mole fractions of the polymer;

PEG_n is a polyethylene glycol polymer and n is an integer from 1 to 2500;

R^1, if present, is any terminal group other than a primary amine;

R^2 is a lower alkyl group;

R^3, if present, is a functional group or linked modifying agent; and

G^1 and G^2 are each independently selected from a polymer segment, a terminal group, a linker and a linked modifying agent.

[0182] Item 2. The thermoreversible polymer according to Item 1, wherein the molecular weight (MW) of the polymer is from about 0 to about 250 kilodaltons (kDa) or about 50 to about 200 kDa.

[0183] Item 3. The thermoreversible polymer of Item 1 or 2, wherein R^1 is a C_1-C_6 alkoxy selected from methoxy, ethoxy, n-propoxy, n-butoxy, isobutoxy, tert-butoxy, pentoxy and isopentoxy.

[0184] Item 4. The thermoreversible polymer of Item 3, wherein R^1 is methoxy.

[0185] Item 5. The thermoreversible polymer of any one of Items 1 to 4, wherein R^2 is selected from the group consisting of methyl, ethyl, propyl, butyl, pentyl, isopropyl, isobutyl, isopentyl, tert-butyl, cyclopropyl, and cyclobutyl.

[0186] Item 6. The thermoreversible polymer of Item 5, wherein R^2 is butyl.

[0187] Item 7. The thermoreversible polymer of any one of Items 1 to 6, wherein R^3 is not present.

[0188] Item 8. The thermoreversible polymer of Item 1, wherein R^1 is methoxy, R^2 is butyl, and R^3 is not present and wherein the MW of the polymer is from about 0 to about 250 kDa.

[0189] Item 9. The thermoreversible polymer of any one of Items 1 to 8, wherein the MW of PEG_n is from about 1 to about 50 kilodaltons (kDa).

[0190] Item 10. The thermoreversible polymer of any one of Items 1 to 9, wherein R^3 is a chemoselective functional group selected from a thiol, an alkyne, a cyclooctyne, an azide, a phosphine, a maleimide, an alkoxyamine, an aldehyde and protected versions or precursors thereof.

[0191] Item 11. The thermoreversible polymer of any one of Items 1 to 9, wherein R^3 is a modifying agent selected from a heparin, a hyaluronic acid, a specific binding member, a peptide, a nucleic acid, gelatin, fibronectin, collagen, laminin, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin, progesterone, glucose, stromal cell derived factor-1 (SDF-1), thymosin beta-4, sonic hedgehog (SHH), Noggin, Activin, transforming growth factor-β (TGF-β), FGF8, brain-derived neurotrophic factor (BDNF), glial cell-derived

neurotrophic factor (GDNF), neurotrophic factor-3 (NT3), platelet-derived growth factor (PDGF), IL-16, IL-2 and insulin-like growth factor-1 (IGF-1).

[0192] Item 12. The thermoreversible polymer of any one of Items 1 to 11, wherein the thermoreversible polymer has one or more of the following properties: (a) an LCST of about 12-32 °C, preferably of about 19-23 °C; (b) a stiffness of about 100 to 8000 Pa, preferably of about 100 to 3000 Pa; and a (c) a viscosity of about 100-1500 cP.

[0193] Item 13. A three-dimensional hydrogel comprising a thermoreversible polymer of any one of Items 1-12, and a buffered aqueous solution.

[0194] Item 14. An *in vitro* method for producing a cell population enriched for MGE progenitor cells from an initial population of human stem cells, the method comprising:

(a) encapsulating an initial population of human stem cells in the three-dimensional hydrogel of Item 13; and

(b) contacting the encapsulated human stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling and with at least one Wingless (Wnt) antagonist; and contacting the cells with at least one activator of Sonic Hedgehog (SHH) signaling and an FGFR agonist to obtain a cell population enriched in MGE progenitor cells expressing FOXG1 and at least one additional marker indicating an MGE progenitor cell.

[0195] Item 15. The method of Item 14, wherein said human stem cells are selected from the group consisting of human embryonic stem cells, human adult stem cells, human neural stem cells, human induced pluripotent cells, human primary progenitor cells, and human induced progenitor cells.

[0196] Item 16. The method of Item 14, wherein said contacting with the at least one inhibitor of SMAD signaling and said contacting with the at least one Wnt antagonist are carried out simultaneously or sequentially, and each has a duration between about 5 days and about 30 days.

[0197] Item 17. The method of Item 16, wherein the contact of the cells with the at least one Wnt antagonist is initiated within 5 days, preferably within 4 days, 3 days, 2 days, or 1 day, from the initial contact of the cells with the at least one inhibitor of SMAD signaling, preferably wherein the contact of the cells with the at least one Wnt antagonist is initiated simultaneously with the initial contact of the cells with the at least one inhibitor of SMAD signaling.

[0198] Item 18. The method of Item 14, wherein said at least one inhibitor of SMAD signaling is selected from the group consisting of SB431542, LDN-193189, Noggin PD169316, SB203580, LY364947, A77-01, A-83-01, BMP4, GW788388, GW6604, SB-505124, lerdelimumab, metelimumab, GC-I008, AP-12009, AP-110I4, LY550410, LY580276, LY364947, LY2109761, SB-505124, E-616452 (RepSox ALK inhibitor), SD-208, SMI6, NPC-30345, KÏ26894, SB-203580, SD-093, activin-M108A, P144, soluble TBR2-Fc, DMH-1, Dorsomorphin dihydrochloride, derivatives thereof, and combinations thereof. .

[0199] Item 19. The method of Item 18, wherein the at least one inhibitor of SMAD signaling comprises SB431542 and LDN-193189.

[0200] Item 20. The method of Item 14, wherein the at least one Wnt antagonist is selected from the group consisting of XAV939, DKK1, DKK-2, DKK-3, Dkk-4, SFRP-1, SFRP-2, SFRP-5, SFRP-3, SFRP-4, WIF-1, Soggy, IWP-2, IWR1, ICG-001, KY0211, Wnt-059, LGK974, IWP-L6, derivatives thereof, and combinations thereof, preferably wherein the at least one Wnt antagonist comprises IWP-2.

[0201] Item 21. The method of Item 14, wherein said at least one activator of SHH signaling is selected from the group consisting of Smoothened agonist (SAG), SAG analog, SHH, C25-SHH, C24-SHH, purmorphamine, Hg—Ag, derivatives thereof, and combinations thereof.

[0202] Item 22. The method of Item 21, wherein (i) the contact of the cells with the at least one activator of SHH signaling is concluded from about 5 days to about 30 days from its initiation, preferably about 18 to 23 days from its initiation, more preferably about 19 to 22 days from its initiation, even more preferably about 20 or 21 days from its initiation; (ii) the initial contact of the cells with the at least one activator of SHH signaling is between about 0 days and

about 10 days, preferably is about 0 days, from the initial contact of the cells with the at least one inhibitor of SMAD signaling and from the initial contact of the cells with the at least one inhibitor of WNT signaling; (iii) the initial contact of the cells with the at least one inhibitor of SMAD signaling is between 0 to 4 days from the initial contact of the cells with the at least one Wnt antagonist; (iv) the contact of the cells with the at least one inhibitor of SMAD signaling is concluded between 6 and 14 days from its initiation and/or (v) the contact of the cells with the at least one Wnt antagonist is concluded between 6 and 8 days from its initiation, preferably about 7 days from its initiation.

[0203] Item 23. The method of Item 14, wherein said at least one additional marker is selected from the group consisting of NKX2-1, NKX2-2, ASCL1, SIX6, OLIG2, NKX6.2, DLX1/2 and LXH6.

[0204] Item 24. The method of Item 14, wherein at least about 60%, at least 65%, at least 70%, at least 75%, or at least 80% of the obtained cell population expresses FOXG1 and NKX2-1.

[0205] Item 25. The method of Item 14, wherein at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80% of the obtained cell population comprises MGE progenitor cells.

[0206] Item 26. The method of Item 14, further comprising (c) contacting the cells, after a predetermined amount of time, with at least one neurotrophic factor (e.g., GDNF, BDNF) and optionally a notch inhibitor (e.g., DAPT) to produce a cell population enriched for differentiated inhibitory GABAergic cortical interneurons (cINs) expressing FOXG1 and at least one additional marker indicating a cIN cell.

[0207] Item 27. The method of Item 26, comprising contacting the cells with at least one neurotrophic factor and a notch inhibitor.

[0208] Item 28. The method of Item 27, comprising contacting the cells with GDNF, BDNF and DAPT.

[0209] Item 29. The method of any one of Items 26-28, wherein contacting the cells with at least one neurotropic factor and optionally a notch inhibitor occurs upon conclusion of contacting the cells with the at least one inhibitor of SMAD signaling and at least one Wnt antagonist and at least one activator of SHH signaling.

[0210] Item 30. The method of any one of Items 26-29, wherein contacting the cells with at least one neurotropic factor and optionally a notch inhibitor is concluded between 7 days and 30 days after initiation and/or contacting the cells with at least one neurotropic factor and optionally a notch inhibitor is concluded at least about 10, at least about 12, or at least about 14 days after initiation.

[0211] Item 31. The method of Item 26, wherein said at least one additional marker is selected from the group consisting of PV, SST, Calbindin, DCX, ASCL1, TUJ1, GABA, GAD1, VGAT, vGLUT1, and GAD67.

[0212] Item 32. The method of any one of Items 26-31, wherein at least about 50%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80% of the obtained cIN cell population expresses parvalbumin (PV).

[0213] Item 33. The method of any one of Items 26-32 wherein less than about 5% of the obtained cIN cell population expresses Ki67.

[0214] Item 34. A composition comprising a population of cells produced by the method of any one of Items 14-26, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells, or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the cells are MGE progenitor cells, preferably wherein the method does not comprise a step to purify or further enrich MGE progenitor cells following step (b).

[0215] Item 35. A composition comprising a population of cells produced by the method of any one of Items 17-33, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells, or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the

cells are cINs, preferably wherein the method does not comprise a step to purify or further enrich cIN cells following step (c).

[0216] Item 36. The composition of Item 35, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells, or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the cells are NKX2.1^{-}/PV^{+}.

[0217] Item 37. The use of a composition according to any one of Items 34-36 in the treatment of a neurological disorder.

[0218] Item 38. The use according to Item 37, wherein the neurological disorder is a seizure disorder.

[0219] Item 39. An *in vitro* method for producing a cell population enriched for MGE progenitor cells from an initial population of human stem cells, the method comprising:

(a) encapsulating an initial population of human stem cells in a three-dimensional hydrogel; and

(b) contacting the encapsulated human stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling and with at least one Wingless (Wnt) antagonist; and contacting the cells with at least one activator of Sonic Hedgehog (SHH) signaling and an FGFR agonist to obtain a cell population enriched in MGE progenitor cells expressing FOXG1 and at least one additional marker indicating an MGE progenitor cell.

[0220] Item 40. The method of Item 39, wherein said human stem cells are selected from the group consisting of human embryonic stem cells, human adult stem cells, human neural stem cells, human induced pluripotent cells, human primary progenitor cells, and human induced progenitor cells.

[0221] Item 41. The method of Item 39, wherein said contacting with the at least one inhibitor of SMAD signaling and said contacting with the at least one Wnt antagonist are carried out simultaneously or sequentially, and each has a duration between about 5 days and about 30 days.

[0222] Item 42. The method of Item 41, wherein the contact of the cells with the at least one Wnt antagonist is initiated within 5 days, preferably within 4 days, 3 days, 2 days, or 1 day, from the initial contact of the cells with the at least one inhibitor of SMAD signaling, preferably wherein the contact of the cells with the at least one Wnt antagonist is initiated simultaneously with the initial contact of the cells with the at least one inhibitor of SMAD signaling.

[0223] Item 43. The method of Item 39, wherein said at least one inhibitor of SMAD signaling is selected from the group consisting of SB431542, LDN-193189, Noggin PD169316, SB203580, LY364947, A77-01, A-83-01, BMP4, GW788388, GW6604, SB-505124, lerdelimumab, metelimumab, GC-I008, AP-12009, AP-110I4, LY550410, LY580276, LY364947, LY2109761, SB-505124, E-616452 (RepSox ALK inhibitor), SD-208, SMI6, NPC-30345, Kï26894, SB-203580, SD-093, activin-M108A, P144, soluble TBR2-Fc, DMH-1, Dorsomorphin dihydrochloride, derivatives thereof, and combinations thereof.

[0224] Item 44. The method of Item 43, wherein the at least one inhibitor of SMAD signaling comprises SB431542 and LDN-193189.

[0225] Item 45. The method of Item 39, wherein the at least one Wnt antagonist is selected from the group consisting of XAV939, DKK1, DKK-2, DKK-3, Dkk-4, SFRP-1, SFRP-2, SFRP-5, SFRP-3, SFRP-4, WIF-1, Soggy, IWP-2, IWR1, ICG-001, KY0211, Wnt-059, LGK974, IWP-L6, derivatives thereof, and combinations thereof, preferably wherein the at least one Wnt antagonist comprises IWP-2.

[0226] Item 46. The method of Item 39, wherein said at least one activator of SHH signaling is selected from the group consisting of Smoothened agonist (SAG), SAG analog, SHH, C25-SHH, C24-SHH, purmorphamine, Hg—Ag, derivatives thereof, and combinations thereof.

[0227] Item 47. The method of Item 46, wherein (i) the contact of the cells with the at least one activator of SHH signaling is concluded from about 5 days to about 30 days from its initiation, preferably about 18 to 23 days from its initiation, more preferably about 19 to 22 days from its initiation, even more preferably about 20 or 21 days from its initiation; (ii) the initial contact of the cells with the at least one activator of SHH signaling is between about 0 days and

about 10 days, preferably is about 0 days, from the initial contact of the cells with the at least one inhibitor of SMAD signaling and from the initial contact of the cells with the at least one inhibitor of WNT signaling; (iii) the initial contact of the cells with the at least one inhibitor of SMAD signaling is between 0 to 4 days from the initial contact of the cells with the at least one Wnt antagonist; (iv) the contact of the cells with the at least one inhibitor of SMAD signaling is concluded between 6 and 14 days from its initiation and/or (v) the contact of the cells with the at least one Wnt antagonist is concluded between 6 and 8 days from its initiation, preferably about 7 days from its initiation.

[0228] Item 48. The method of Item 39, wherein said at least one additional marker is selected from the group consisting of NKX2-1, NKX2-2, ASCL1, SIX6, OLIG2, NKX6.2, DLX1/2 and LXH6.

[0229] Item 49. The method of Item 39, wherein at least about 60%, at least 65%, at least 70%, at least 75%, or at least 80% of the obtained cell population expresses FOXG1 and NKX2-1.

[0230] Item 50. The method of Item 39, wherein at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80% of the obtained cell population comprises MGE progenitor cells.

[0231] Item 51. The method of Item 39, further comprising (c) contacting the cells, after a predetermined amount of time, with at least one neurotrophic factor (e.g., GDNF, BDNF) and optionally a notch inhibitor (e.g., DAPT) to produce a cell population enriched for differentiated inhibitory GABAergic cortical interneurons (cINs) expressing FOXG1 and at least one additional marker indicating a cIN cell.

[0232] Item 52. The method of Item 51, comprising contacting the cells with at least one neurotrophic factor and a notch inhibitor.

[0233] Item 53. The method of Item 52, comprising contacting the cells with GDNF, BDNF and DAPT.

[0234] Item 54. The method of any one of Items 51-53, wherein contacting the cells with at least one neurotropic factor and optionally a notch inhibitor occurs upon conclusion of contacting the cells with the at least one inhibitor of SMAD signaling and at least one Wnt antagonist and at least one activator of SHH signaling.

[0235] Item 55. The method of any one of Items 51-54, wherein contacting the cells with at least one neurotropic factor and optionally a notch inhibitor is concluded between 7 days and 30 days after initiation and/or contacting the cells with at least one neurotropic factor and optionally a notch inhibitor is concluded at least about 10, at least about 12, or at least about 14 days after initiation.

[0236] Item 56. The method of Item 51, wherein said at least one additional marker is selected from the group consisting of PV, SST, Calbindin, DCX, ASCL1, TUJ1, GABA, GAD1, VGAT, vGLUT1, and GAD67.

[0237] Item 57. The method of any one of Items 51-56, wherein at least about 50%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80% of the obtained cIN cell population expresses parvalbumin (PV).

[0238] Item 58. The method of any one of Items 51-57 wherein less than about 5% of the obtained cIN cell population expresses KI67.

[0239] Item 59. A composition comprising a population of cells produced by the method of any one of Items 39-50, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells, or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the cells are MGE progenitor cells, preferably wherein the method does not comprise a step to purify or further enrich MGE progenitor cells following step (b).

[0240] Item 60. A composition comprising a population of cells produced by the method of any one of Items 51-58, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells, or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the

cells are cINs, preferably wherein the method does not comprise a step to purify or further enrich cIN cells following step (c).

[0241] Item 61. The composition of Item 60, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells, or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the cells are $NKX2.1^{-}/PV^{+}$.

[0242] Item 62. The use of a composition according to any one of Items 59-61 in the treatment of a neurological disorder.

[0243] Item 63. The use according to Item 62, wherein the neurological disorder is a seizure disorder.

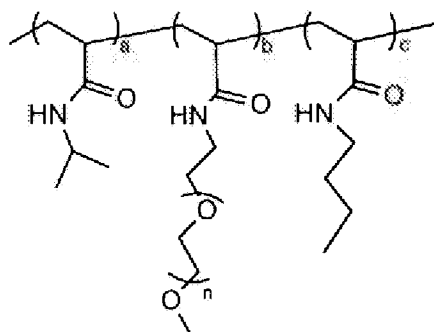
EXAMPLES

[0244] The following examples illustrate preferred embodiments of the present invention and are not intended to limit the scope of the invention in any way. While this invention has been described in relation to its preferred embodiments, various modifications thereof will be apparent to one skilled in the art from reading this application.

Example 1

[0245] The production of cINs from human stem cells requires multiple factors, careful timing, and long maturation periods. As such, three-dimensional hydrogels should remain stable over these relatively long time periods.

[0246] Hydrogels disclosed in U.S. Patent Publication No. 2024/0294713 comprising acrylamide polymers of the following structure were initially tested for, *inter alia*, stability during the duration of cIN production from human stem cells:

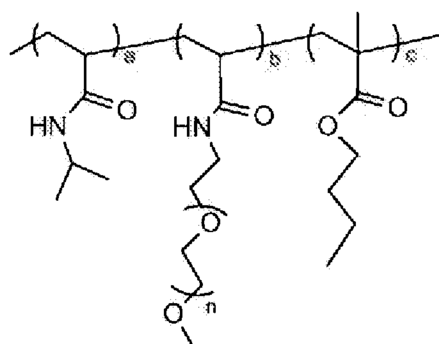


[PNIPAAm-*co*-PBAm]-*b*-[PEG]

[0247] The production of cINs from human stem cells was not possible with hydrogels comprising such acrylamide polymers because the hydrogel was not stable over the full 35-day differentiation period, at any molecular weight tested (from 0 to 500 kDa). These hydrogels also did not have a suitable peak stiffness range for stability (<1200 Pa), exhibited high viscosity (>2000 cP), had low stability/high swell ratio in aqueous environments(>1.5), and high LCSTs (>25). These gels did not hold structure in aqueous environments over time, and did not hold structure with the shear stress of mixing in spinner flasks or bioreactors. Finally, these gels did not extrude and encapsulate cells well, with low sphericity (<0.7), large bead diameters (>4mm), and large fraction of tail formation (>0.5).

[0248] As such, structural improvements for increased stability were tested.

[0249] It was first determined that utilizing acrylate in the polymers (see process in Fig. 1) having the following structure:



[0250] **[PNIPAAm-*co*-PBMA]-*b*-[PEG]**

corresponding to formula III, wherein R^1 is methoxy, R^2 is butyl, R^4 is methyl and (d) is not present, and MW of the polymer is 250-500 kDa, resulted in decreased LCST, increased gel stiffness, lowered viscosity and increased gel stability relative to acrylamide .

The results are shown in Table 1 below and Figure 2:

Table 1

Parameter	**Acrylate**	**Acrylamide**
MW Range	**250-500 kDa**	**250-500 kDa**
LCST	12-32 C	24-32 C
Stiffness	100-4000 Pa	100-2000 Pa
Viscosity	2000-5000 cP	4000-7000 cP
Stability	Medium stability	Low stability

[0251] Next, the properties of acrylate polymers of the structure above were modified to further improve the stability of hydrogels over time. Polymer backbones of various molecular weights were produced by altering the initiator concentration. It was determined that lowering the molecular weight of the overall polymer (a function of the length of the backbone which directly controls the length of the polymer, with a reduction in backbone length corresponding to a reduction in molecular weight of the polymer) increases stiffness, maintains the LCST, decreases liquid phase viscosity and increases stability (all functional groups remained as defined above); The results are shown in Table 2 below and Figure 3:

Table 2

Parameter	Low MW (5-50 kDa)	Medium MW (50-250 kDa)	High MW (250-500 kDa)
LCST	12-32 C	12-32 C	12-32 C
Stiffness	100-1200 Pa	100-8000 Pa	100-4000 Pa
Viscosity	100-1000 cP	100-2000 cP	2000-5000 cP
Stability	Low Stability	High Stability	Medium Stability

[0252] Thus, it was determined that three-dimensional hydrogels comprising low molecular weight, acrylate-based polymers provide significant improvements over prior art thermoreversible polymers for, e.g., three-dimensional culture of stem cells to produce cINs. In particular, three-dimensional hydrogels comprising low molecular weight acrylate-based polymers exhibit increased stability, enabling the lengthy differentiation process required to produce cINs, decreased liquid phase viscosity enabling efficient cell encapsulation,, and an appropriate stiffness that supports neural growth, while maintaining advantageous LCST.

[0253] The effect of varying the molecular weight of PEG in polymers of formula III on properties of 3-dimensional hydrogels was tested. It was surprisingly determined that PEG lengths of 1k or lower result in poor gel performance at a matched wt% (15wt%), while PEG lengths of 10k or higher hinder gel flexibility and node stability. As such, PEG weight of about 5k was determined to be optimal. See figure 4.

[0254] Next, varying the effect of the weight ratio of PEG to polymer backbone in polymers of formula III was tested. It was discovered that increasing the ratio of PEG to PNIPAAm above 1:2 enables improvements to synthesis and performance in encapsulation and cell growth. See Table 3 below and Figure 5A,

Table 3

	1-2 wt ratio PEG:PNIPAAm	**1-3 wt ratio PEG:PNIPAAm**	**1-4 wt ratio PEG:PNIPAAm**

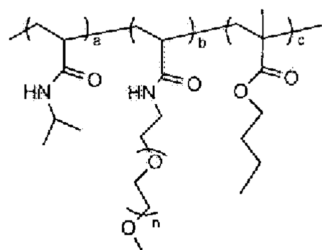
Polymer	Stringy	Powdery	Powdery
Opacity in Water	Hazy	Clear	Clear
Sterile Filtering	Slow	Medium	Fast
Viscosity	High	Low	Low
Stiffness	Medium	High	High
Opacity in Gel	Transparent	Opaque	Opaque
Encapsulated Beads	~40 uL	~20 uL	~20 uL
Gel Debris	Tails Present	No Debris	No Debris
Gel Stability	Medium	High	High

[0255] Increasing the weight ratio of PEG to PNIPAAm above 1:2 increases gel stiffness, lowers gel viscosity, increases gel stability and lowers gel LCT. See Figure 5A. Without being bound by theory, these advantages may derive from increased PNIPAAm participation in the node formation of the gel, and stronger associations to support temperature-based gel formation.

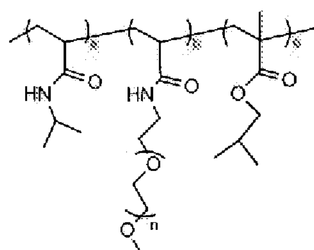
[0256] Increasing the ratio of PEG to PNIPAAm copolymer above 1:2 also enables improvements to cell encapsulation, as the encapsulated beads exhibit decreased bead diameter, decreased bead volume, increased bead sphericity and decrease in the fraction of beads containing tails after gravity drip encapsulation. See Figure 5B.

[0257] Additional improvements when the weight ratio of PEG to PNIPAAm is above 1:2 include improvements to encapsulation and cell performance, such as faster flow rates with lower shear stress, higher immediate cell viability, higher 7 day cell viability and higher cell yield. See Figure 5C.

[0258] Next, the effect of the alkyl group at position R^2 of formula III was investigated. Briefly, the following polymers of formula III (R^1 = methoxy, R^4 = methyl, (d) = 0 and R^2 is n-butyl or isobutyl) were prepared and tested:



[PNIPAAm-*co*-PBMA]-*b*-[PEG]



[PNIPAAm-*co*-PIBMA]-*b*-[PEG]

[0259] The conformation of the pendent alkyl group (n-butyl vs isobutyl) was determined to be significant, with n-butyl surprisingly exhibiting higher stiffness with similar viscosity, higher gel stability and lower gel LCST compared to isobutyl. See Fig. 6.

[0260] The conformation of the backbone acrylate group was determined to be important, as methyl at position R4 of formula III (from butyl methacrylate) exhibits higher stiffness with similar viscosity, higher gel stability, and lower gel LCST compared to hydrogen at position R4. See Figures 6.5A-6.5B. Moreover, polymers of formula III having methyl at position R4 exhibit smaller bead diameter and volume, higher bead sphericity, and similar bead tail fraction formation. See Figure 6.5C.

[0261] Polymers of formula III as described herein comprise a combination of features rendering them uniquely useful for encapsulating and suspending cells in three-dimensional hydrogel bioreactors for scale-up production in tanks for lengthy cell culture/differentiation protocols such as production of functional neurons from stem cells. The present hydrogels (comprising encapsulated differentiated cells such as neurons) can be used for therapeutic purposes in humans. The present systems maintain the viability of cells (e.g., stem cells) and avoid harsh methods of recovery of the cells from the system.

[0262] Polymers of formula III can be functionalized multiple positions including pendant and backbone positions. For example, functionalization may be achieved by adding monoamine-PEG terminated with the respective functional groups as in the following. Polymers of formula

III with the following groups at pendant position R^1 were created: R^1 = methoxy; R^1 = hydroxyl; R^1 = acrylate; R^1 = biotin; R^1 = DBCO. See Fig. 7.

[0263] Polymers of formula III with the following groups at backbone position R^3 were created: R^3 = methacrylate; R^3 = maleimide; R^3 = DBCO. See Fig. 8 in which functionalization was achieved by reacting free NASI groups with amine-conjugated functional groups before isopropyl amine saturation.

[0264] To demonstrate the ability of polymers of formula III to bind and release proteins in the context of stem cell differentiation, polymers of formula III with acrylate at position R^1 were reacted with thiol-proteins (FGF and heparin) by thiol-Michael addition. See Fig. 9.

[0265] Surprisingly, the pendant PEG group (at position R^1) was found to significantly affect synthesis of the polymer. In particular, groups at position R^1 other than primary amines enables higher reaction v/v%, higher yield per volume of solvent, and more reproducible synthesis. See Fig. 10A. Producing an amine terminated R^1 requires the use of a bifunctional diaminoPEG monomer in the synthesis, which has the potential for unintended covalent crosslinking. This can greatly affect polymer performance, reproducibility, and viscosity, and may prevent the polymer from reliquifying. To attempt to avoid this, synthesis with diaminoPEG requires very dilute reactions (<2 w/v%) during synthesis, and limits the polymer yield per unit volume in scale-up while presenting batch-to-batch variability. Synthesis with monoamine-PEG eliminates the potential for unintentional crosslinking, enabling more reproducible synthesis and enables synthesis scale-up.

[0266] Further, the solvent choice for reaction 1 was found to be critical for successful polymer synthesis and incorporation of each monomer at the intended molar ratio and for precise control of the molecular weights. Certain solvents with the ideal solubility properties for all monomers exhibit high reaction efficiencies with complete incorporation (A), while some solvents only polymerize monomers that are soluble, not all monomers (B), and some solvents inhibit complete polymerization of the reaction (C-D), and some polymers are not suitable for any reaction (E-F). Solvent tested ranged from polar protic solvents, polar aprotic solvents, and

nonpolar solvents in standard and anhydrous forms in various structural conformations. Tested solvents include Acetone, Acetonitrile, Benzene, Chloroform, Dichloromethane, Dimethylformamide, Dimethyl sulfoxide, Dioxane, Ethyl acetate, Pyridine, Ethanol, Methanol, Tetrahydrofuran, Toluene, and Water. See Fig. 10B

Example 2

[0267] Several alternative neural induction protocols were evaluated for the production of post-mitotic cIN precursor cells from human stem cells in a three-dimensional hydrogel, with varying media formulations and timing of addition of different components.

[0268] In particular, culture protocols described in the following references were assessed for a 35 day differentiation process: (1) Maroof *et al.*, Cell Stem Cell, 12(5):559-572 (2013); (2) Nicholas *et al.*, Cell Stem Cell, 12(5):573-586 (2013); and (3) Kim *et al.*, Stem Cells, 32(7):1789-1804 (2014), the entire contents of each which is incorporated herein by reference. An alternative without wnt inhibition was tested for each protocol. An alternative replacing SB for AZD (ERK inhibitor) was tested for the Studer protocol.

[0269] According to Maroof *et al.*, dual SMAD and wnt inhibition occurs for 9 days, SHH activation occurs for 8 days and no final specification/maturation factors are added:

Days	0-9	10-17	18-32	Transplantation D32
Factors	LDN/ SB / XAV939	PPA / SHH	No Patterning Factors	
Media	DMEM/KSR/Gluta/BME	DMEM / Gluta / N2 / B27	Neurobasal / Gluta / B27	

[0270] According to Nicholas *et al.*, dual SMAD and wnt inhibition occurs for 14 days, SHH activation occurs for 35 days, and BDNF/DAPT are added for 11 days:

	Suspension EBs (+RI)	Attached EBs		Monolayer Culture	
Days	0-6	7-14	15-25	25-35	Transplantation D35
Factors	SB / LDN (Instead of BMPRIA) / XAV939 (Instead of DKK1) / PPA		PPA	PPA+BDNF+DAPT	
Media	Neurobasal-A / B27-vitA / NEAA / BME / Gluta			Neurobasal-A / NEAA / BME / Gluta	

[0271] According to Kim *et al.*, dual SMAD and wnt inhibition occurs for 7 days, FGF8 is added for 14 days, SHH activation occurs for 21 days and BDNF/GDNF/DAPT are added for 14 days:

	Low Adherent EBs			Monolayer Culture	
Days	0-7	8-14	15-21	22-35	Transplantation D35
Factors	SB/LDN/IWP2/SAG	LDN/SAG/FGF8	SAG/FGF8	BDNF/GDNF/DAPT	
Media	DMEM/KSR/Gluta/BME			DMEM-F12/N2	

[0272] At day 18, results using the neural induction protocols were evaluated by assessing the generation of MGE (Figure 11A). As shown in Figures 11B-E, by day 18, MGE progenitor cells characterized by expression of FoxG1, NKX2-1 and DLK1/2 were efficiently generated in a three-dimensional hydrogel only using the Kim *et al.* protocol.

[0273] At day 35, results using the neural induction protocols were evaluated by assessing the generation of cortical interneurons (Figure 12A). As shown in Figures 12B-F, by day 35, MGE-derived cortical interneurons or cortical interneuron precursor cells, characterized by expression of FoxG1, Calbindin, Gad1 and downregulation of Nkx2-1, were efficiently generated using the Kim *et al.* protocol. Notably, although not as efficient as the Kim *et al.* protocol, the Maroof et al. protocol tested in the three-dimensional hydrogel was vastly superior to the original protocol performed in two-dimensional cell culture (see Figures 12A-E). Further, all media alternatives without Wnt inhibition or by replacing SB by AZD were less efficient at generating MGE progenitors.

Example 3

[0274] Proof-of-concept studies were performed utilizing a low molecular weight, acrylate backbone 3D hydrogel to demonstrate robust derivation of encapsulated hPSCs into post-mitotic cINs with therapeutic potential to treat neurological disorders such as epilepsy.

[0275] Methods

[0276] ***Preparation of 3-dimension PEG-PNIPAAM hydrogel*** A two-step synthesis process was used to produce a thermoreversible graft copolymer (Figure 1), where the PEG represents the hydrophilic block, the PNIPAAm represents the hydrophobic block, and the alkyl pendant group (here described as butyl chains but could encompass any alkyl chain) serves as the temperature shifting moiety. To generate the thermoreversible graft copolymer, a mixture of NIPAAm, N-acryloxysuccinimide (NASI), and an alkyl-chain methacrylate was first copolymerized via standard radical polymerization. The resulting functionalizable copolymer, after reprecipitation and drying, was then mixed with a monoamine-terminated PEG block. The amine-terminated group attached to the PNIPAAm-co-PNASI-co-MA backbone via the amine and N-hydroxysuccinimide (NHS) amidation reaction. Finally, the remaining NHS groups were converted to PNIPAAm via addition of isopropylamine, and the resulting polymer was dried, dialyzed, and lyophilized.

[0277] ***hPSC Expansion***

[0278] Human PSC cells (H9 human embryonic stem cells (WA09, WiCell, Madison, WI, passage 53-58)), were maintained on Matrigel (BD, San Jose, CA) in E8 media (Gibco, Billings, MT) and passaged using Versene (Thermo Fisher, Waltham, MA), following manufacturer's recommendation.

[0279] ***Cell Encapsulation, Differentiation and Harvest***

[0280] For differentiation, hPSCs were dissociated with Accutase (Stem Cell Technologies, Vancouver, BC, Canada) and encapsulated using PEG-PNIPAAM hydrogel at a 10%wt/v final, using a concentration of 250,000 cells/ml of gel. Hydrogel and cells were mixed on ice and 50 ul droplets were subsequently generated and extruded into well plates as shown in Figure 13b, for a

total of 5 gel droplets per well. Plates were incubated at 37°C for 15 min to allow for gelification, forming dome-shaped beads. Complete E8 media heated to 37°C supplemented with Rock Inhibitor Y-27632 (Selleck Chemicals, Houston, TX) was subsequently added (5% v/v media/gel final) and cells were incubated at 37°C with 5% CO2. Fifty percent of the media was replaced daily making sure that the plates remained above 33°C. After 48hr in expansion conditions, culture media was replaced by differentiation media containing DMEM, knockout serum replacement (KSR, 20%), 2mM L-glutamine and 10 μM β-mercaptoethanol (all from Thermo Fisher). For neural induction, cells were treated with LDN193189 (100 nM, Stemgent, Cambridge, MA) from D0 to D14 and with SB431542 (10 μM, Tocris, Minneapolis, MN) from D0 to D7. For MGE induction, cells were treated with IWP2 (5 μM, Selleck Chem) from D0 to D7, with SAG (0.1 μM, XcessBio, Chicago, IL) from D0 to D21, and with FGF8 (100ng/ml, Peprotech) from D8 to D21. On D22, media was replaced by DMEM F/12 (Stem Cell Technologies) containing 10ng/ml GDNF (R&D Systems Minneapolis, MN), 10ng/ml BDNF (R&D) and 2.5μM DAPT (Tocris) for further differentiation and maturation. At the indicated timepoints, aggregates were dissociated to single cells in the presence of Accumax (Innovative Cell Technologies, San Diego, CA) and TrypLE (Thermo Fisher) in a rotating platform.

[0281] ***Cell Counting and Viability Analysis***

[0282] Cells were stained with AOPI and counted using the K2 image capture equipment and Matrix software (PerkinElmer, Waltham, MA), to obtain the total cell count and percentage viability.

[0283] ***Flow Cytometry***

[0284] Differentiated cells were dissociated and fixed in CytoFix/CytoPerm solution (BD) for 20 minutes and washed with Perm/Wash (BD). For staining, cells were incubated with primary antibodies for 30 minutes. After washing with Perm/Wash, Alexa 647-conjugated secondary antibodies (Thermo Fisher) were added and incubated for another 30 minutes. After washing with Perm/Wash, cells were resuspended in PBS and analyzed using an Attune NxT Flow Cytometer (Thermo Fisher). NovoExpress (Agilent, Santa Clara, CA) software was used to analyze raw data. Ten thousand events were used per analysis.

[0285] *Immunocytochemistry*

[0286] For immunofluorescence staining, fixed cells were incubated with Intercept Blocking Buffer (LI-COR Biosciences, Lincoln, NE), for 30 minutes and permeabilized using 0.25% Triton-x for 10 minutes. Cells were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. After rinsing with PBS, samples were incubated with fluorescent dye-labeled secondary antibodies (Alexa 488 or Alexa 647-labeled IgG; Thermo Fisher) and Hoechst 33342 (4 mg/ml) in blocking buffer for 1 hour at room temperature. After rinsing with PBS, imaging was performed using a Cytation 5 imaging system (Agilent) and images were analyzed using FIJI image analysis software (Schindelin, J. *et al.* Fiji: An open-source platform for biological-image analysis. *Nature Methods* vol. 9 Preprint at https://doi.org/10.1038/nmeth.2019 (2012)).

[0287] *Quantitative PCR*

[0288] Total RNA was prepared using RNeasy kit (Qiagen, Germantown, MD) and cDNA from total RNA was generated using the RT2 First Strand kit (Qiagen). For quantitative analysis of transcript expression, real-time PCR analyses were performed using the RT2 SYBR Green qPCR Mastermixes (Qiagen) and an AriaMX Real Time PCR System (Agilent). Primers were designed using the Integrated DNA Technologies PrimerQuest tools (Coralville, IA). The mRNA expression level for each gene was normalized against that of the ACTB gene. The relative values were calculated by setting the normalized value of control as 1.

[0289] Results

[0290] ***Encapsulation of human pluripotent stem cells into thermoreversible hydrogels for differentiation into cortical interneurons***

[0291] The copolymer hydrogel utilized herein is based on hydrophilic poly(ethylene glycol) (PEG) and temperature-sensitive poly(N-isopropylacrylamide) (PNIPAAm). Upon heating above the lower critical solution temperature (LCST), the PNIPAAm component becomes increasingly hydrophobic and enables micelle formation, essentially physical "crosslinking" the PEG-

PNIPAAm polymer (Figure 13a). This hydrogel can be used to encapsulate and differentiate hPSCs in scale down (well plate) models as well as in stirring cultures including perfused stirred-tank bioreactors, by simply extruding hydrogel droplets containing single cells or small cell clusters into warm culture media, leading to the formation of gel capsules containing these cells (Figures 13b and c). This encapsulation method allows for a fully defined, synthetic, xeno-free and scalable platform for manufacturing of clinically relevant cell types.

[0292] The feasibility of differentiating human pluripotent stem cells (hPSCs) into GABAergic cortical interneuron precursor cells encapsulated in the hydrogel was tested using a well-established media (Kim, T. G. *et al.* Efficient specification of interneurons from human pluripotent stem cells by dorsoventral and rostrocaudal modulation. *Stem Cells* **32,** (2014)) (Figure 13d). Briefly, human embryonic stem cells (WA09) were seeded in hydrogel beads and allowed to expand for 48 hours in minimal proliferation media (Gibco-E8) supplemented with ROCK inhibitor (Y-27632) to maximize cell viability as described previously (Watanabe, K. *et al.* A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* **25,** (2007)). To promote differentiation of hPSCs into neuroectodermal lineages, the ALK2/3 inhibitor LDN193189 and ALK5/7 inhibitor SB431542 were used, as described previously (Kim, T. G. *et al.* Efficient specification of interneurons from human pluripotent stem cells by dorsoventral and rostrocaudal modulation. *Stem Cells* **32,** (2014)). In addition, a chemical inhibitor of Wnt pathway, IWP2, was used to promote rostralization of early neuroectoderm and subsequently to inhibit dorsalization of neuroectoderm (Kim, T. G., 2014). An activator of Sonic Hedgehog (SHH) pathway (SAG) was used to define the MGE identity over LGE. To counteract the caudalizing effect of SHH activation, FGF8 was added to shift the balance towards rostralization, efficiently generating MGE progenitors (Kim, T. G., 2014). Finally, to promote further differentiation of MGE progenitors into cINs and further neural maturation, glial cell line derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and γ-secretase inhibitor DAPT were added.

[0293] To monitor patterning efficacy into ventral telencephalon, MGE progenitors and cIN progenitor cells, previously described markers that characterize these stages were used for quantitative PCR (qPCR), flow cytometry (FC) and immunocytochemistry (ICC) (Figure 13e)

(Marín, O. Human cortical interneurons take their time. *Cell Stem Cell* vol. 12 Preprint at https://doi.org/10.1016/j.stem.2013.04.017 (2013); Kim, T. G. *et al.* Efficient specification of interneurons from human pluripotent stem cells by dorsoventral and rostrocaudal modulation. *Stem Cells* **32,** (2014); Nicholas, C. R. *et al.* Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development. *Cell Stem Cell* **12,** (2013); Cunningham, M. *et al.* hPSC-derived maturing GABAergic interneurons ameliorate seizures and abnormal behavior in epileptic mice. *Cell Stem Cell* **15,** (2014); and Maroof, A. M. *et al.* Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. *Cell Stem Cell* **12,** (2013)).

[0294] ***Efficient differentiation of hPSCs into GABAergic cortical interneurons in three-dimensional hydrogel platform***

[0295] Flow cytometry analysis allowed us to determine cell identity and purity at different timepoints throughout differentiation. By day 10 of differentiation of hPSCs encapsulated in three-dimensional hydrogels, these cells started to express FoxG1, an early marker of ventral telencephalon progenitor cells (Figures 14a and b). By day 18, an average of 88.44% of the cells expressed FoxG1, and 78.4% expressed the MGE marker NKX2-1, indicating a strong patterning into telencephalon-derived MGE progenitor cells (Figures 14a and b). Upon further maturation, by day 35, cells exhibited high expression of FoxG1 (78.14%) and significant downregulation of NXK2-1 down to 22.52%, compatible with a mature post-mitotic cIN phenotype (Figures 14a and b). To confirm the MGE progenitor and cIN phenotypes, qPCR and ICC analysis were performed (Figure 51). qPCR analysis shows high expression of ventral telencephalon and MGE progenitor markers FoxG1 and DLX2 by day 18 compared to undifferentiated hPSCs, as well as upregulation of SST, suggesting early induction of genes characterizing MGE-derived mature cIN population (Figure 15a). By day 35 of differentiation, cells exhibited upregulation of cIN-like genes such as GAD1 (glutamic acid decarboxylase, involved in GABA biosynthesis), Calbindin (CALB1) and further upregulation of SST (Figure 15a). Consistent with this qPCR analysis, ICC analysis at day 35 showed a high percentage of cells expressing FoxG1, compatible with the MGE origin of these cells, the neural cell adhesion protein NCAM, high synthesis of GABA (85.1% GABA+) as well as the mature cIN markers PV (84.5% PV+) and

SST (42.3% SST+). In addition, ki67 was below 1%, indicating that the majority of these cells are indeed post-mitotic (Figures 15b and c).

[0296] ***Thermoreversible hydrogel culture platform renders higher viability and differentiation efficacy of hPSCs into GABAergic cortical interneurons compared to standard two-dimensional culture methods***

[0297] We next sought to compare the efficacy of the synthetic 3D hydrogel platform for cIN production with a standard 2D method (Maroof, A. M. *et al.* Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. *Cell Stem Cell* **12,** (2013)). We first compared post-harvest viability. After 35 days of differentiation, cells harvested from 3D hydrogels grown as cellular aggregates exhibited high (>80%) viability, whereas cells harvested from two-dimensional cultures exhibited low viability (~20%) (Figures 16a and b). This indicated that the gentler harvest and dissociation method from the thermoreversible hydrogel provided a substantial improvement on cell viability upon harvest compared to standard 2D cultures. Furthermore, we evaluated the differentiation efficacy of cells encapsulated in the novel hydrogel of formula (III) compared to cells manufactured in 2D. We found that the percentage of cells expressing FoxG1 was ~800 fold higher in 3D compared to 2D by day 10 (Figure 16c). By day 18, the percentage of FoxG1+ cells was ~100-fold higher in 3D compared to 2D and NK2X-1 expression was ~10 fold higher compared to 2D (Figure 15c). By day 35, we obtained ~100-fold higher FoxG1 expression in 3D compared to 2D (Figure 16c). Altogether, these findings demonstrate a higher differentiation efficacy into cINs when hPSCs are encapsulated and grown as aggregates in a 3D synthetic hydrogel compared to standard 2D methods.

[0298] ***Discussion***

[0299] We have demonstrated that hPSCs can be robustly differentiated into MGE progenitor cells and cINs when encapsulated in three-dimensional hydrogels, rendering over 80% expression of MGE progenitor marker FoxG1 and over 75% expression of Nkx2-1 by day18, followed by over 75% FoxG1 expression by day 35 with high percentage of cells expressing cIN markers PV, SST, GAD1 and GABA, together with a strong downregulation in Nkx2-1

expression to only ~22%. This, together with nearly absent ki67 expression, indicates that a 3D thermoreversible hydrogel platform allows for efficient generation of mature post-mitotic cINs.

[0300] Furthermore, a side-by-side comparison between a standard 2D cell production methodology and the synthetic 3D hydrogel process using the same media profile shows a stunning superiority of the synthetic 3d hydrogel culture platform, with~800-fold higher percentage of FoxG1+ cells by day 18 and ~100-fold higher percentage of FoxG1+ cells by day 35. Together with this, the gentle harvest process accounted for ~4 times higher viability compared to cell detachment from 2D platforms. Functional differentiation and maturation of hPSCs into cIN *in vitro* has been suggested to occur over a protracted period, resembling their lengthy maturation process during human brain development (Marín, O. Human cortical interneurons take their time. *Cell Stem Cell* vol. 12). Interestingly, marker expression analysis of cells harvested at day 35 from the novel hydrogel of formula (III) suggests an accelerated maturation timeframe compared to what has been reported so far using traditional culture methods. Nkx2-1 downregulation has been reported after 5 weeks of *in vivo* grafting of 5-week old cells (Kim, T. G. *et al.* Efficient specification of interneurons from human pluripotent stem cells by dorsoventral and rostrocaudal modulation. *Stem Cells* **32,** (2014)), after 30 weeks of differentiation (98% to 66% expression (Nicholas, C. R. *et al.* Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development. *Cell Stem Cell* **12,** (2013)), or 4 months post-transplant of 3-week old cells, seeing a downregulation in NKX2 to ~29% (Zhu, Q. *et al.* Human cortical interneurons optimized for grafting specifically integrate, abort seizures, and display prolonged efficacy without over-inhibition. *Neuron* (2023) doi:10.1016/j.neuron.2022.12.014). In addition to this, SST expression was only detected after 20-30 weeks of differentiation (~12-40%) (Nicholas C.R. et al., Ibid) or 5 months post grafting (Kim T.G. et al., Ibid).

[0301] Regarding PV expression, just 10% of cells expressing this protein was reported at 15 weeks of differentiation (Nicholas C.R. et al., Id) or after 4-5 months post grafting *in vivo* (8-10%) (Kim T.G. et al., Ibid; Zhu, Q. et al., Id). In contrast, utilizing the present methods, 84.5% PV+ cINs were detected on Day 35. It has been reported before that culturing NKX2-1+ MGE progenitor cells on mouse cortical extracts leads to an accelerated maturation timeframe into

cINs rendering detection of PV expression by day 30 of differentiation *in vitro*. This suggests that the presence of certain cell-cell interactions and/or signaling molecules are key to accelerating this maturation process, although the mechanism involved has not been identified (Maroof, A. M. *et al.* Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. *Cell Stem Cell* **12,** (2013)). Without being bound by theory, the methods described herein may promote cell-cell interactions as well as boost autocrine and paracrine signaling by trapping key signaling molecules secreted by these cells within the hydrogel *milieu* that otherwise are washed away in traditional 2D cultures or 3D suspension platforms. Altogether, the data present herein demonstrates robust differentiation of cINs from hPSCs in 3D synthetic hydrogels such as the novel hydrogel of formula (III), with substantially higher viability and differentiation efficacy compared to standard 2D cultures, as well as accelerated differentiation period compared to the maturation timeframe reported using standard 2D cultures or hybrid 2D-suspension systems.

Example 4

[0302] Proof-of-concept studies were performed utilizing a low molecular weight, acrylate backbone 3D hydrogel (of formula III) to demonstrate robust expansion of encapsulated hPSCs.

[0303] Methods

[0304] ***Preparation of 3-dimension PEG-PNIPAAM hydrogel*** A two-step synthesis process was used to produce a thermoreversible graft copolymer (Figure 1), where the PEG represents the hydrophilic block, the PNIPAAm represents the hydrophobic block, and the alkyl pendant group (here described as butyl chains but could encompass any alkyl chain) serves as the temperature shifting moiety. To generate the thermoreversible graft copolymer, a mixture of NIPAAm, N-acryloxysuccinimide (NASI), and an alkyl-chain methacrylate was first copolymerized via standard radical polymerization. The resulting functionalizable copolymer, after reprecipitation and drying, was then mixed with a monoamine-terminated PEG block. The amine-terminated group attached to the PNIPAAm-co-PNASI-co-MA backbone via the amine and N-hydroxysuccinimide (NHS) amidation reaction. Finally, the remaining NHS groups were

converted to NIPAAm via addition of isopropylamine, and the resulting polymer was dried, dialyzed, and lyophilized.

[0305] ***hPSC Expansion***

[0306] Human PSC cells (H9 human embryonic stem cells (WA09, WiCell, Madison, WI, passage 53-58)), were maintained on Matrigel (BD, San Jose, CA) in E8 media (Gibco, Billings, MT) and passaged using Versene (Thermo Fisher, Waltham, MA) or ReleSR (Stem Cell Technologies, Vancouver, BC, Canada), following manufacturer's recommendation.

[0307] ***Cell Encapsulation, Differentiation and Harvest***

[0308] For expansion, hPSCs were dissociated with Accutase (Stem Cell Technologies, Vancouver, BC, Canada) and encapsulated using PEG-NIPAAM hydrogel at 2.5-10%wt/v final, using a concentration of 250,000 cells/ml of gel. Hydrogel and cells were mixed ,encapsulated and extruded into vessels of different sizes (100mL to 1L). Complete E8 media heated to 37°C supplemented with Rock Inhibitor Y-27632 (Selleck Chemicals, Houston, TX) was subsequently added and cells were incubated at 37°C with 5% CO2. At the end of the expansion process (7 days) aggregates were dissociated to single cells in the presence of Accumax (Innovative Cell Technologies, San Diego, CA) and TrypLE (Thermo Fisher) in a rotating platform.

[0309] ***Cell Counting and Viability Analysis***

[0310] Cells were stained with AOPI and counted using the K2 image capture equipment and Matrix software (PerkinElmer, Waltham, MA), to obtain the total cell count and percentage viability.

[0311] ***Flow Cytometry***

[0312] Cells were dissociated and fixed in CytoFix/CytoPerm solution (BD) for 20 minutes and washed with Perm/Wash (BD). For staining, cells were incubated with primary antibodies for 30 minutes. After washing with Perm/Wash, cells were resuspended in PBS and analyzed

using an Attune NxT Flow Cytometer (Thermo Fisher). NovoExpress (Agilent, Santa Clara, CA) software was used to analyze raw data. Ten thousand events were used per analysis.

[0313] *Quantitative PCR*

[0314] Total RNA was prepared using RNeasy kit (Qiagen, Germantown, MD) and cDNA from total RNA was generated using the RT2 First Strand kit (Qiagen). For quantitative analysis of transcript expression, real-time PCR analyses were performed using the RT2 SYBR Green qPCR Mastermixes (Qiagen) and an AriaMX Real Time PCR System (Agilent). Primers were designed using the Integrated DNA Technologies PrimerQuest tools (Coralville, IA). The mRNA expression level for each gene was normalized against that of the ACTB gene. The relative values were calculated by setting the normalized value of control as 1.

[0315] Discussion

[0316] This gel formulation allowed for the generation of beads comprised of core and shell geometries that allow for maximal cell retention upon scale up (Fig 5D). 3 different culture scales are used to quickly iterate through different conditions in scale down models that closely predict the performance in bioreactors (positive displacement pipettes – PDP - static and spinners, Fig 17A). Utilizing this improved formulation, we demonstrated high viability upon encapsulation. High-viability throughout the encapsulation process allowed for scalable hPSC expansion, with encapsulation flow rates of a minimum of 2 ml gel/min. Example expansion processes utilizing this formulation are shown on Fig 18B, showing reproducible, high-quality, hPSC expansion across 4 different bioreactor scales (Fig 18A), from 100mL to 1L vessel volumes, as validated by flow cytometry (Fig 18B) and qPCR (18C). In addition, robust expansion in this hydrogel was shown with both a human embryonic stem cell line (hESC) as well as a human induced pluripotent stem cell (hiPSC) line, as shown in 17B, 17C.

Example 5

Proof-of-concept studies were performed utilizing a low molecular weight, acrylate backbone 3D hydrogel to demonstrate robust derivation of encapsulated hPSCs into pancreatic endoderm progenitor (PE) cells.

[0317] Methods

[0318] ***Preparation of 3-dimension PEG-PNIPAAM hydrogel*** A two-step synthesis process was used to produce a thermoreversible graft copolymer (Figure 1), as described for Example 4.

[0319] ***hPSC Expansion***

[0320] Human PSC cells (H9 human embryonic stem cells (WA09, WiCell, Madison, WI, passage 53-58)), were maintained on Matrigel (BD, San Jose, CA) in E8 media (Gibco, Billings, MT) and passaged using Versene (Thermo Fisher, Waltham, MA) or ReleSR (Stem Cell Technologies, Vancouver, BC, Canada), following manufacturer's recommendation.

[0321] ***Cell Encapsulation, Differentiation and Harvest***

[0322] For differentiation, hPSCs were dissociated with Accutase (Stem Cell Technologies, Vancouver, BC, Canada) and encapsulated using PEG-PNIPAAM hydrogel at a 2.5-10%wt/v final, using a concentration of 250,000 cells/ml of gel. Hydrogel and cells were mixed ,encapsulated and extruded into 100mL spinners. Spinners were incubated at 37°C for 15 min to allow for gelification. Complete E8 media heated to 37°C supplemented with Rock Inhibitor Y-27632 (Selleck Chemicals, Houston, TX) was subsequently added and cells were incubated at 37°C with 5% CO_2. Fifty percent of the media was replaced daily making sure that the plates remained above 33°C. After 48hr in expansion conditions, culture media was replaced by differentiation media following manufacturer recommendations (STEMdiff, Definitive Endoderm, Stem Cell Technologies, Vancouver, BC, Canada) (Fig 20A). At the indicated timepoints (Fig 19), aggregates were dissociated to single cells in the presence of Accumax (Innovative Cell Technologies, San Diego, CA) and TrypLE (Thermo Fisher) in a rotating platform.

[0323] ***Cell Counting and Viability Analysis***

[0324] Cells were stained with AOPI and counted using the K2 image capture equipment and Matrix software (PerkinElmer, Waltham, MA), to obtain the total cell count and percentage viability.

[0325] *Flow Cytometry*

[0326] Cells were dissociated and fixed in CytoFix/CytoPerm solution (BD) for 20 minutes and washed with Perm/Wash (BD). For staining, cells were incubated with primary antibodies for 30 minutes. After washing with Perm/Wash, cells were resuspended in PBS and analyzed using an Attune NxT Flow Cytometer (Thermo Fisher). NovoExpress (Agilent, Santa Clara, CA) software was used to analyze raw data. Ten thousand events were used per analysis.

[0327] Discussion

[0328] Increased gel stability allowed for longer differentiation processes and full vessel exchange volumes without compromising gel integrity, both key to ensure high differentiation efficacy upon scale up. As shown in Fig 19, PE cells were produced in this hydrogel utilizing an off-the-shelf media formulation in the improved 3D hydrogel system and compared to the standard process in suspension. As a result, 45-fold higher PE production was obtained compared to 3D suspension (standard cultures), higher aggregate size control, high viability post-inoculation (>90%) and expression of PE progenitor cell marker PDX-1 (Figs 20B-D). Of note, the higher aggregate size control unlocked by this 3D culture method allows for the generation of aggregates below 500 um. This demonstrates that hydrogels comprising a thermoreversible polymer of formula III has broad implications in clinical manufacturing as aggregates surpassing this size can exhibit necrotic cores due to nutrient limitation and/or suboptimal differentiation efficacy due to heterogeneous response to differentiation cues throughout the aggregate.

Example 6

[0329] Proof-of-concept studies were performed utilizing a low molecular weight, acrylate backbone 3D hydrogel to demonstrate robust derivation of encapsulated hPSCs into midbrain dopaminergic cells (mDAs)

[0330] Methods

[0331] ***Preparation of 3-dimension PEG-PNIPAAM hydrogel*** A two-step synthesis process was used to produce a thermoreversible graft copolymer (Figure 1), as described for Example 4.

[0332] ***hPSC Expansion***

[0333] Human PSC cells (H9 human embryonic stem cells (WA09, WiCell, Madison, WI, passage 53-58)), were maintained on Matrigel (BD, San Jose, CA) in E8 media (Gibco, Billings, MT) and passaged using Versene (Thermo Fisher, Waltham, MA) or ReleSR (Stem Cell Technologies, Vancouver, BC, Canada), following manufacturer's recommendation.

[0334] ***Cell Encapsulation, Differentiation and Harvest***

[0335] For differentiation, hPSCs were dissociated with Accutase (Stem Cell Technologies, Vancouver, BC, Canada) and encapsulated using PEG-PNIPAAM hydrogel at a 2.5-10%wt/v final, using a concentration of 500,000 cells/ml of gel. Hydrogel and cells were mixed and plated with positive displacement pipettes ("PDP") in plates or encapsulated and extruded into 100mL spinners. Plates were incubated at 37°C for 15 min to allow for gelification, forming dome-shaped beads. Complete E8 media heated to 37°C supplemented with Rock Inhibitor Y-27632 (Selleck Chemicals, Houston, TX) was subsequently added and cells were incubated at 37°C with 5% CO2. Fifty percent of the media was replaced daily making sure that the plates remained above 33°C. After 48hr in expansion conditions, culture media was replaced by differentiation media as described previously (Adil et al., *Sci Rep* 7, 40573 (2017)) (Fig 21A). On day 16 of differentiation (Fig 21A), aggregates were dissociated to single cells in the presence of Accumax (Innovative Cell Technologies, San Diego, CA) and TrypLE (Thermo Fisher) in a rotating platform.

[0336] ***Cell Counting and Viability Analysis***

[0337] Cells were stained with AOPI and counted using the K2 image capture equipment and Matrix software (PerkinElmer, Waltham, MA), to obtain the total cell count and percentage viability.

[0338] ***Flow Cytometry***

[0339] Cells were dissociated and fixed in CytoFix/CytoPerm solution (BD) for 20 minutes and washed with Perm/Wash (BD). For staining, cells were incubated with primary antibodies for 30 minutes. After washing with Perm/Wash, cells were resuspended in PBS and analyzed using an Attune NxT Flow Cytometer (Thermo Fisher). NovoExpress (Agilent, Santa Clara, CA) software was used to analyze raw data. Ten thousand events were used per analysis.

[0340] Discussion

[0341] Improved gel stability allowed for longer differentiation processes and full vessel exchange volumes without compromising gel integrity, both key to ensure high differentiation efficacy upon scale up. As shown in Fig 21B and C, mDAs were produced in the improved 3D hydrogel system. High differentiation efficacy was achieved at different scales (PDP and 100mL spinner), measured by FoxA2 expression (70-90%), a marker for ventral midbrain progenitor cells, with high viability upon harvest (>80%) (Figs 21B and C). This data indicates that the improved hydrogel formulation is compatible with scalable culture systems, with high gel stability throughout lengthy differentiation processes, protecting cells from shear stress produced by bioreactor mixing.

Example 7

[0342] Proof-of-concept studies were performed utilizing a low molecular weight, acrylate backbone 3D hydrogel to demonstrate robust derivation of encapsulated hPSCs into hematopoietic stem cells (HSCs) as well as expansion of primary HSC cells.

[0343] Methods

[0344] ***Preparation of 3-dimension PEG-PNIPAAM hydrogel*** A two-step synthesis process was used to produce a thermoreversible graft copolymer (Figure 1), as described for Example 4.

[0345] ***hPSC Expansion***

[0346] Human PSC cells (H9 human embryonic stem cells (WA09, WiCell, Madison, WI, passage 53-58)), were maintained on Matrigel (BD, San Jose, CA) in E8 media (Gibco, Billings, MT) and passaged using Versene (Thermo Fisher, Waltham, MA) or ReleSR (Stem Cell Technologies, Vancouver, BC, Canada), following manufacturer's recommendation.

[0347] ***Cell Encapsulation, Differentiation and Harvest***

[0348] For HSC differentiation, hPSCs were dissociated with Accutase (Stem Cell Technologies, Vancouver, BC, Canada) and encapsulated using PEG-PNIPAAM hydrogel at a 2.5-10%wt/v final, using a concentration of 500,000 cells/ml of gel. Hydrogel and cells were mixed and plated with positive displacement pipettees ("PDP") in plates. Plates were incubated at 37°C for 15 min to allow for gelification, forming dome-shaped beads. Complete E8 media heated to 37°C supplemented with Rock Inhibitor Y-27632 (Selleck Chemicals, Houston, TX) was subsequently added and cells were incubated at 37°C with 5% CO2. Fifty percent of the media was replaced daily making sure that the plates remained above 33°C. After 48hr in expansion conditions, culture media was replaced by differentiation media following manufacturer's recommendations for the StemDiff Hematopoietic kit (Stem Cell Technologies, Vancouver, BC, Canada) (Fig 22A). On day 12 of differentiation (Fig 22A), cells were harvested by cooling the hydrogel to 4C. For HSC expansion, primary human cord blood CD34+ HSCs (Stem Cell Technologies, Vancouver, BC, Canada) were thawed and encapsulated in the hydrogel as described above and seeded utilizing PDP method . Cells were expanded in SFEM II media supplemented with StemSpan CD34+ expansion supplement for 8 days (Fig 23A). Cells were subsequently harvested by cooling the hydrogel to 4C.

[0349] ***Cell Counting and Viability Analysis***

[0350] Cells were stained with AOPI and counted using the K2 image capture equipment and Matrix software (PerkinElmer, Waltham, MA), to obtain the total cell count and percentage viability.

[0351] Discussion

[0352] Improved gel stability allowed for longer differentiation processes and full vessel exchange volumes without compromising gel integrity, both key to ensure high differentiation efficacy upon scale up. As shown in Fig 22B and C, hPSCs were differentiated into HSCs utilizing the improved 3D hydrogel system. In addition, human cord blood CD34+ HSCs were also successfully expanded in this 3D hydrogel system (Fig 23B and C). This data indicates that the improved hydrogel formulation is compatible with HSC expansion and differentiation from hPSCs, with high gel stability throughout lengthy differentiation processes, protecting cells from shear stress produced by bioreactor mixing upon scale up. HSCs are a promising cell therapy candidate with broad therapeutic applications from sickle cell disease and b thalassemia to blood cancers.

Example 8

[0353] Comparison of hydrogels comprising thermoreversible polymers of formula III with hydrogels comprising thermoreversible polymers disclosed in US Patent No. 10,982,055.

[0354] Synthesis of thermoreversible polymers as disclosed in US Patent No. 10,982,055 is shown at Figure 3 of US 10,982,055. Synthesis of thermoreversible polymers of formula III of the present disclosure is shown at Fig 1. The following table summarizes key differences in synthesis of the respective polymers:

Table 4

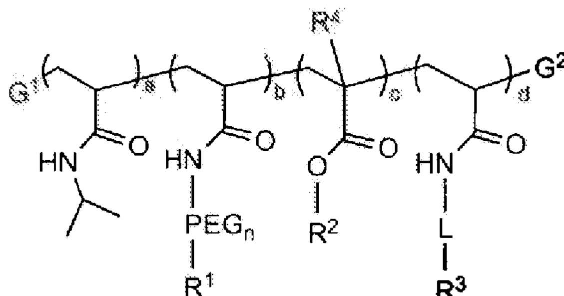
	US Pat. No. 10,982,055	**Formula III**

Polymerization Approach	React in specific environment to achieve specific molecular weights	React in optimal environment for high reaction efficiency and reproducibility
Molecular Weight Ranges Created	6-46 kDa	5-500 kDa
Reaction 1 Monomers	NASI and iso-butyl MA	NIPAAm, NASI, and n-butyl MA
Polymerization Solvent	Mix of Toluene/Benzene	Singular optimized solvent
Molecular Weight Control	Bulk solvent solubility (mixing different solvents)	Initiator concentration (reacting to completion)
PEG Binding	PEG diamine	PEG monoamine
Potential for Unintentional Covalent Crosslinking	Yes, due to diamine must react lower than 2% v/v to try and limit	No, monoamine cannot crosslink, can react up to 15% v/v
Temperature Shifting Component	iso-butyl MA (less hydrophobic)	n-butyl MA (more hydrophobic)
Ability to Reliquefy	Sometimes	Always
Performance	Low peak stiffness Low stability	High peak stiffness High stability
Synthesis Reproducibility	Limited	High
Synthesis Scalability	Limited (2% max v/v)	High (15% max v/v)

[0355] While the materials and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the method described herein without departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

CLAIMS

1. A thermoreversible polymer with improved stability over time, comprising the formula (III):



wherein (a), (b), (c), and (d) represent mole fractions of co-monomers in the polymer, wherein (a), (b), and (c) are each greater than 0;

PEG_n is a polyethylene glycol polymer and n is an integer;

R^1 , if present, is any terminal or functional group other than a primary amine;

R^2 is a lower alkyl group;

R^3, if present, is a terminal or functional group or linked modifying agent;

R^4 is hydrogen or a lower alkyl group;

G^1 and G^2 are each independently selected from a polymer segment, a terminal group, a linker and a linked modifying agent,

wherein the molecular weight of the polymer is greater than 50 kDa.

2. The thermoreversible polymer according to claim 1, wherein the molecular weight (MW) of the polymer is from about 50 kDa to about 250 kDa.

3. The thermoreversible polymer according to claim 1 or 2, wherein the PEG_n is a polyethylglycol polymer having a MW of about 1 kDa to about 50 kDa or about 2 kDa to about 20 kDa.

4. The thermoreversible polymer according to any one of claims 1 to 3, wherein the thermoreversible polymer has a weight:weight (w/w) ratio of PEG to PNIPAAm copolymer above about 1:2, preferably wherein the thermoreversible polymer has a weight:weight (w/w) ratio of PEG to PNIPAAm copolymer of about 1:2.5 to about 1:4.5.

5. The thermoreversible polymer of any one of claims 1 to 4, wherein R^1 is absent or is any terminal or functional group other than alkyl or substituted alkyl.

6. The thermoreversible polymer according to any one of claims 1-5, wherein R^1 is C_1-C_6 alkoxy selected from methoxy, ethoxy, n-propoxy, n-butoxy isobutoxy, tert-butoxy, pentoxy and isopentoxy or is hydroxy.

7. The thermoreversible polymer according to claim 6, wherein R^1 is methoxy or wherein R^1 is hydroxyl, biotin, or DBCO.

8. The thermoreversible polymer according to any one of claims 1 to 7, wherein R^2 is other than isobutyl.

9. The thermoreversible polymer according to any one of claims 1 to 7, wherein R^2 is selected from the group consisting of methyl, ethyl, propyl, butyl, pentyl, isopropyl, isobutyl, isopentyl, tert-butyl, cyclopropyl, and cyclobutyl.

10. The thermoreversible polymer according to claim 9, wherein R^2 is n-butyl, iso-butyl, or tera-butyl, preferably wherein R^2 is n-butyl.

11. The thermoreversible polymer according to any one of claims 1 to 10, wherein R^3 is not present.

12. The thermoreversible polymer according to any one of claims 1 to 4, wherein R^1 is methoxy and R^2 is n-butyl, preferably wherein R^4 is methyl.

13. The thermoreversible polymer according to claim 12, wherein (d) is not present.

14. The thermoreversible polymer according to any one of claims 1 to 13, wherein R^3 is absent.

15. The thermoreversible polymer according to any one of claims 1 to 13, wherein R^1 and/or R^3 is a chemoselective functional group selected from acrylate, methacrylate, biotin, streptavidin, thiol, an alkyne, a cyclooctyne, an azide, a phosphine, a maleimide, an alkoxyamine, an aldehyde and protected versions or precursors thereof.

16. The thermoreversible polymer according to any one of claims 1 to 13, wherein R^1 and/or R^3 is a modifying agent selected from a heparin, a hyaluronic acid, a specific binding member, a peptide, a nucleic acid, gelatin, fibronectin, collagen, laminin, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin, progesterone, glucose, stromal cell derived factor-1 (SDF-1), thymosin beta-4, sonic hedgehog (SHH), Noggin, Activin, transforming growth factor-β (TGF-β), FGF8, brain-derived neurotrophic factor (BDNF), glial cell-derived

neurotrophic factor (GDNF), neurotrophic factor-3 (NT3), platelet-derived growth factor (PDGF), IL-16, IL-2 and insulin-like growth factor-1 (IGF-1).

17. The thermoreversible polymer according to any one of claims 1 to 16, wherein the thermoreversible polymer has one or more, and preferably all, of the following properties: (a) an LCST of about 12-32 °C, preferably of about 19-23 °C; (b) a stiffness of about 100 to 8000 Pa; and (c) a viscosity of about 100-2000 cP.

18. A three-dimensional hydrogel comprising a thermoreversible polymer according to any one of claims 1-17, and a buffered aqueous solution.

19. The three-dimensional hydrogel according to claim 18, wherein the hydrogel is stable at 37 C in a buffered environment for a period of at least one week, at least two weeks, at least three weeks, at least four weeks, at least five weeks, at least six weeks, at least seven weeks, or at least eight weeks or at least three months.

20. A composition comprising a plurality of hydrogel capsules, wherein at least 90%, preferably at least 95% of said hydrogel capsules in said composition comprise at least one cell and a hydrogel encapsulating said cell, wherein said hydrogel encapsulating said cell is a hydrogel comprising a thermoreversible polymer according to any one of claims 1-17, and a buffered aqueous solution.

21. The composition according to claim 20, wherein at least 90% of said hydrogel capsules in said composition each comprises at least 100, at least 200, at least 300, at least 400, at least 500, at least 600, at least 700, at least 800 or at least 9000 cells, preferably wherein the cells are stem cells.

22. A method for expanding or producing a population of differentiated cells from a stem cell or a precursor cell, the method comprising culturing the stem cell or precursor cell in a three-dimension hydrogel according to claim 18 or 19 under conditions suitable for inducing differentiation of the stem cell or precursor cell.

23. The method according to claim 22, wherein the conditions suitable for expansion or differentiation of the stem cell or precursor cell comprises culturing the stem cell or precursor cell in the hydrogel composition for a period of at least one week, at least two weeks, at least

three weeks, at least four weeks, at least five weeks, at least six weeks, at least seven weeks, or at least eight weeks or at least three months.

24. An *in vitro* method for producing a cell population enriched for MGE progenitor cells from an initial population of human stem cells, the method comprising:

(a) encapsulating an initial population of human stem cells in the three-dimensional hydrogel according to claim 18 or 19; and

(b) contacting the encapsulated human stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling and with at least one Wingless (Wnt) antagonist; and contacting the cells with at least one activator of Sonic Hedgehog (SHH) signaling and an FGFR agonist to obtain a cell population enriched in MGE progenitor cells expressing FOXG1 and at least one additional marker indicating an MGE progenitor cell.

25. The method of claim 24, wherein said human stem cells are selected from the group consisting of human embryonic stem cells, human adult stem cells, human neural stem cells, human induced pluripotent cells, human primary progenitor cells, and human induced progenitor cells.

26. The method of claim 24, wherein said contacting with the at least one inhibitor of SMAD signaling and said contacting with the at least one Wnt antagonist are carried out simultaneously or sequentially, and each has a duration between about 5 days and about 30 days.

27. The method of claim 26, wherein the contact of the cells with the at least one Wnt antagonist is initiated within 5 days, preferably within 4 days, 3 days, 2 days, or 1 day, from the initial contact of the cells with the at least one inhibitor of SMAD signaling, preferably wherein the contact of the cells with the at least one Wnt antagonist is initiated simultaneously with the initial contact of the cells with the at least one inhibitor of SMAD signaling.

28. The method of claim 24, wherein said at least one inhibitor of SMAD signaling is selected from the group consisting of SB431542, LDN-193189, Noggin PD169316, SB203580, LY364947, A77-01, A-83-01, BMP4, GW788388, GW6604, SB-505124, lerdelimumab, metelimumab, GC-I008, AP-12009, AP-110I4, LY550410, LY580276, LY364947, LY2109761, SB-505124, E-616452 (RepSox ALK inhibitor), SD-208, SMI6, NPC-30345, KÏ26894, SB-

203580, SD-093, activin-M108A, P144, soluble TBR2-Fc, DMH-1, Dorsomorphin dihydrochloride, derivatives thereof, and combinations thereof.

29. The method of claim 28, wherein the at least one inhibitor of SMAD signaling comprises SB431542 and LDN-193189.

30. The method of claim 24, wherein the at least one Wnt antagonist is selected from the group consisting of XAV939, DKK1, DKK-2, DKK-3, Dkk-4, SFRP-1, SFRP-2, SFRP-5, SFRP-3, SFRP-4, WIF-1, Soggy, IWP-2, IWR1, ICG-001, KY0211, Wnt-059, LGK974, IWP-L6, derivatives thereof, and combinations thereof, preferably wherein the at least one Wnt antagonist comprises IWP-2.

31. The method of claim 24, wherein said at least one activator of SHH signaling is selected from the group consisting of Smoothened agonist (SAG), SAG analog, SHH, C25-SHH, C24-SHH, purmorphamine, Hg—Ag, derivatives thereof, and combinations thereof.

32. The method of claim 31, wherein (i) the contact of the cells with the at least one activator of SHH signaling is concluded from about 5 days to about 30 days from its initiation, preferably about 18 to 23 days from its initiation, more preferably about 19 to 22 days from its initiation, even more preferably about 20 or 21 days from its initiation; (ii) the initial contact of the cells with the at least one activator of SHH signaling is between about 0 days and about 10 days, preferably is about 0 days, from the initial contact of the cells with the at least one inhibitor of SMAD signaling and from the initial contact of the cells with the at least one inhibitor of WNT signaling; (iii) the initial contact of the cells with the at least one inhibitor of SMAD signaling is between 0 to 4 days from the initial contact of the cells with the at least one Wnt antagonist; (iv) the contact of the cells with the at least one inhibitor of SMAD signaling is concluded between 6 and 14 days from its initiation and/or (v) the contact of the cells with the at least one Wnt antagonist is concluded between 6 and 8 days from its initiation, preferably about 7 days from its initiation.

33. The method of claim 24, wherein said at least one additional marker is selected from the group consisting of NKX2-1, NKX2-2, ASCL1, SIX6, OLIG2, NKX6.2, DLX1/2 and LXH6.

34. The method of claim 24, wherein at least about 60%, at least 65%, at least 70%, at least 75%, or at least 80% of the obtained cell population expresses FOXG1 and NKX2-1.

35. The method of claim 24, wherein at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80% of the obtained cell population comprises MGE progenitor cells.

36. The method of claim 24, further comprising (c) contacting the cells, after a predetermined amount of time, with at least one neurotrophic factor (e.g., GDNF, BDNF) and optionally a notch inhibitor (e.g., DAPT) to produce a cell population enriched for differentiated inhibitory GABAergic cortical interneurons (cINs) expressing FOXG1 and at least one additional marker indicating a cIN cell.

37. The method of claim 36, comprising contacting the cells with at least one neurotrophic factor and a notch inhibitor.

38. The method of claim 37, comprising contacting the cells with GDNF, BDNF and DAPT.

39. The method of any one of claim 36-38, wherein contacting the cells with at least one neurotropic factor and optionally a notch inhibitor occurs upon conclusion of contacting the cells with the at least one inhibitor of SMAD signaling and at least one Wnt antagonist and at least one activator of SHH signaling.

40. The method of any one of claims 36-39, wherein contacting the cells with at least one neurotropic factor and optionally a notch inhibitor is concluded between 7 days and 30 days after initiation and/or contacting the cells with at least one neurotropic factor and optionally a notch inhibitor is concluded at least about 10, at least about 12, or at least about 14 days after initiation.

41. The method of claim 36, wherein said at least one additional marker is selected from the group consisting of PV, SST, Calbindin, DCX, ASCL1, TUJ1, GABA, GAD1, VGAT, vGLUT1, and GAD67.

42. The method of any one of claims 36-41, wherein at least about 50%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80% of the obtained cIN cell population expresses parvalbumin (PV).

43. The method of any one of claims 36-42 wherein less than about 5% of the obtained cIN cell population expresses Ki67.

44. A composition comprising a population of cells produced by the method of any one of claims 24-36, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells,

or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the cells are MGE progenitor cells, preferably wherein the method does not comprise a step to purify or further enrich MGE progenitor cells following step (b).

45. A composition comprising a population of cells produced by the method of any one of claims 27-43, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells, or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the cells are cINs, preferably wherein the method does not comprise a step to purify or further enrich cIN cells following step (c).

46. The composition of claim 45, wherein at least 50% of the cells, or at least 60% of the cells, or at least 70% of the cells, or at least 80% of the cells, or at least 90% of the cells, or at least 95% of the cells are $NKX2.1^{-}/PV^{+}$.

47. The use of a composition according to any one of claims 44-46 in the treatment of a neurological disorder.

48. The use according to claim 47, wherein the neurological disorder is a seizure disorder.

49. A method preparing a thermoreversible polymer of formula III, the method comprising the steps of (i) polymerizing a comonomer population comprising N-isopropylacrylamide (NIPAAm), alkyl meth/acrylate, and N-acryloxysuccinimide (NASI) in a single solvent in the presence of an initiator concentration effective to form a PNIPAAM-co-PBMA-co-PNASI copolymer backbone having a MW of at least 50 kDa; and (ii) reacting the copolymer with a monoPEGamine to form a [PNIPAAM-co-PBMA-co-PNASI]-b-[PEG] copolymer and (iii) reacting the [PNIPAAM-co-PBMA-co-PNASI]-b-[PEG] with isopropylamine to form a thermoreversible polymer of formula III.

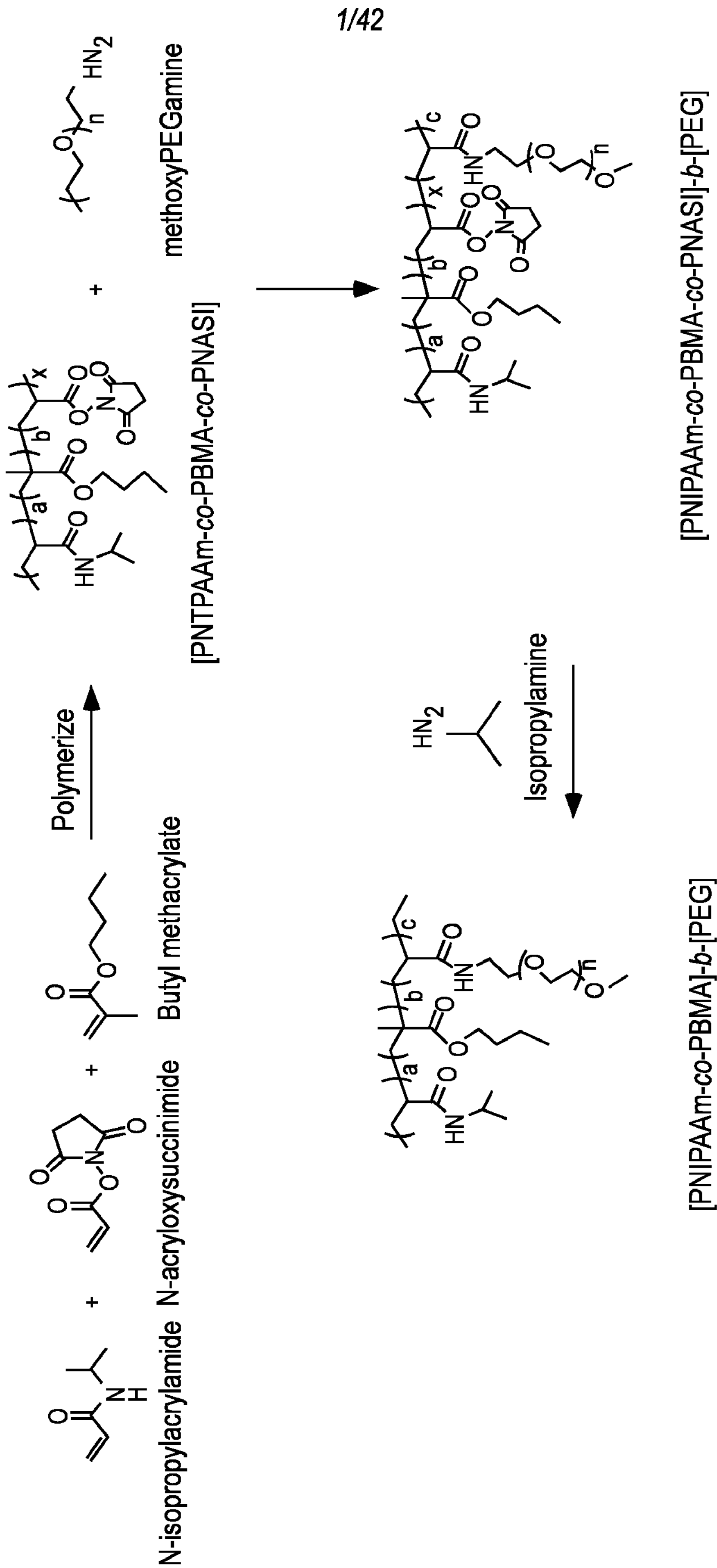
50. The method according to claim 49, wherein the single solvent is selected from Acetone, Acetonitrile, Benzene, Chloroform, Dichloromethane, Dimethylformamide, Dimethyl sulfoxide, Dioxane, Ethyl acetate, Pyridine, Ethanol, Methanol, Tetrahydrofuran, Toluene, and Water.

51. A thermoreversible polymer produced by a method according to claim 49 or 50.

52. A three-dimensional hydrogel comprising a thermoreversible polymer according to claim 51.

53. The thermoreversible polymer of claims 1-17, further comprising expansion of human hematopoietic stem cells for at least a week.

54. The thermoreversible polymer of claims 1-17, further comprising pluripotent stem cells and differentiating these into midbrain dopaminergic cells, pancreatic endoderm cells and hematopoietic stem cells.



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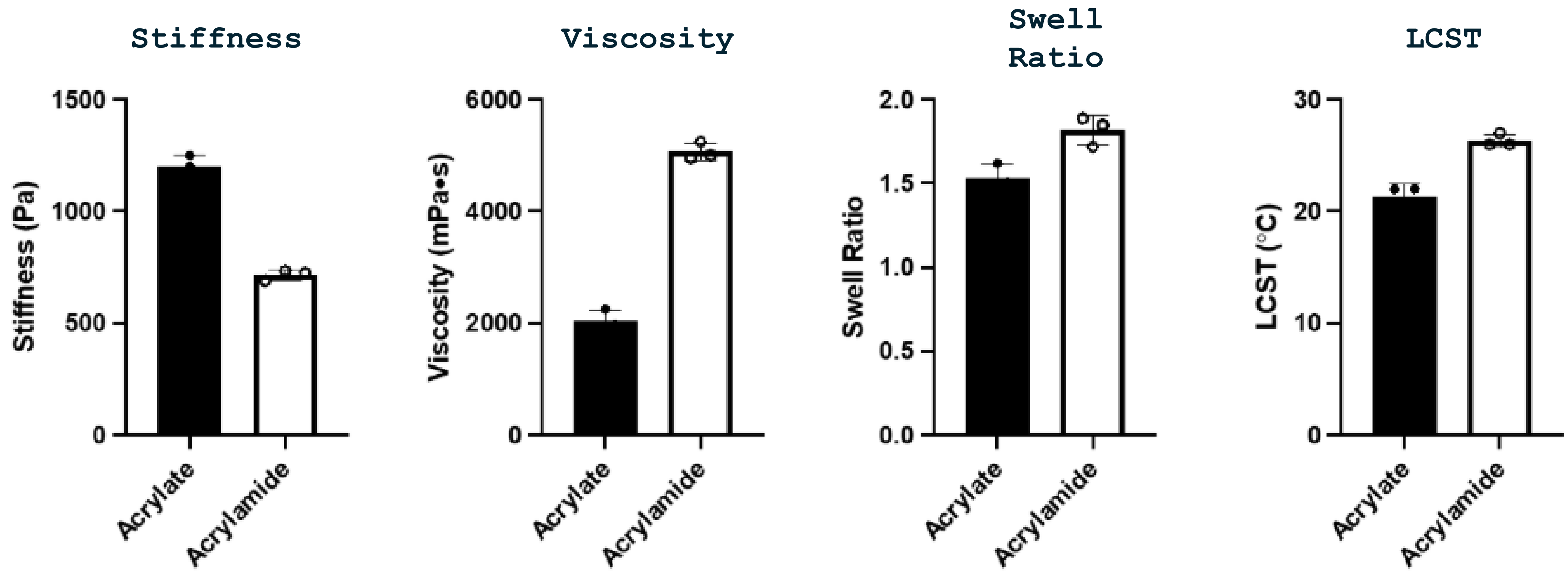


Figure 2

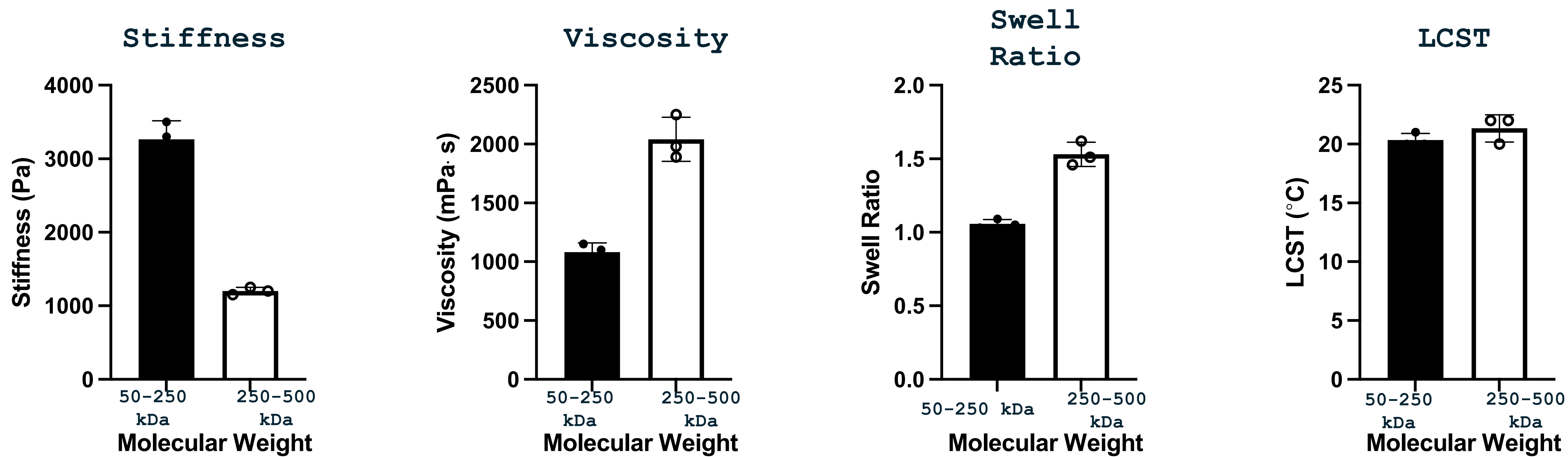


Figure 3

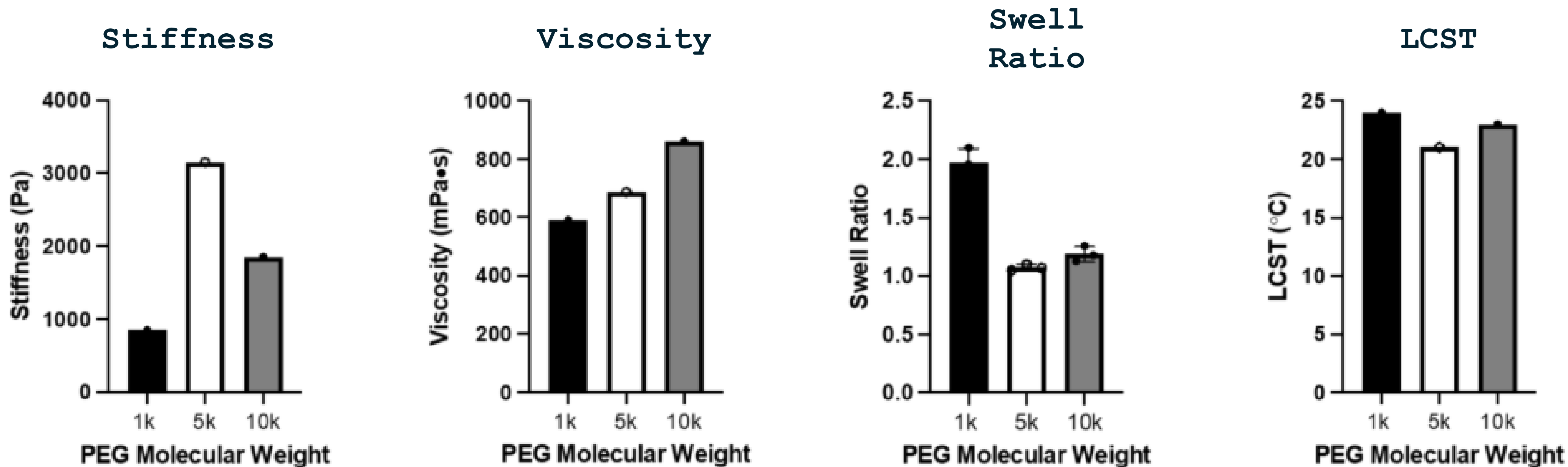


Figure 4

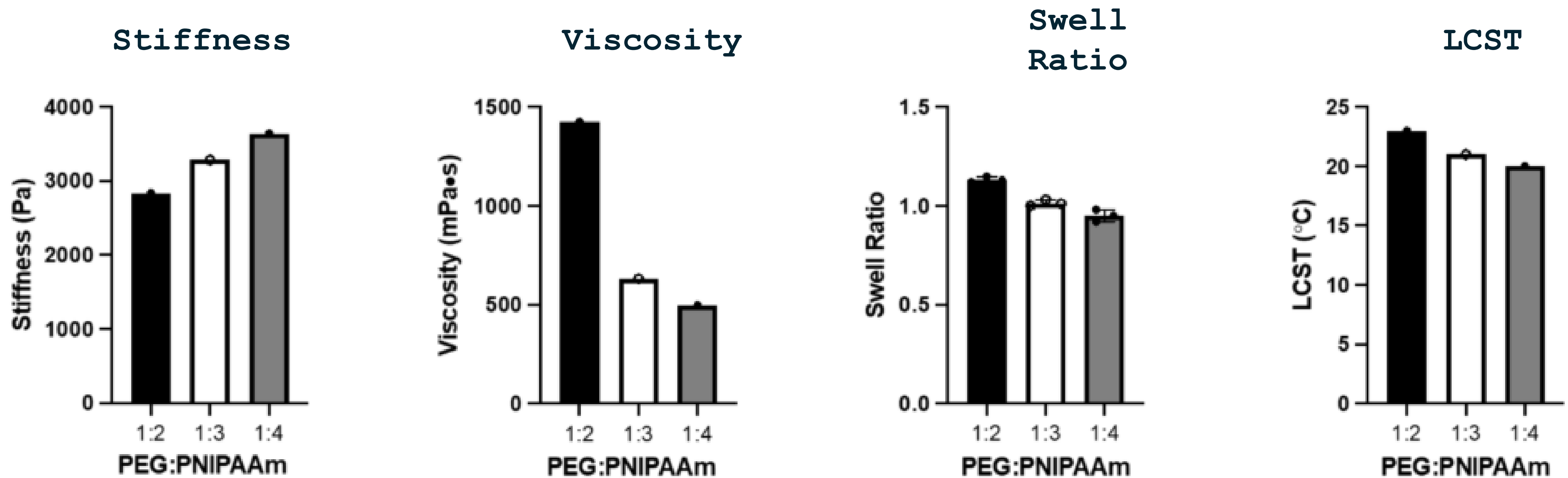


Figure 5A

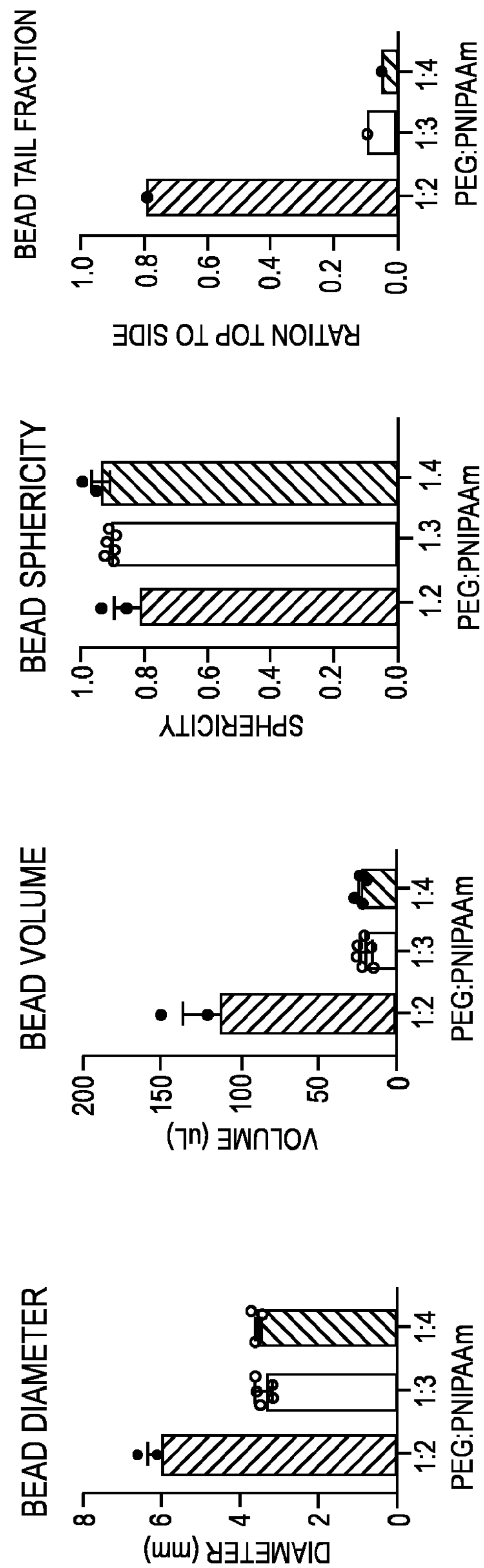


FIG. 5B

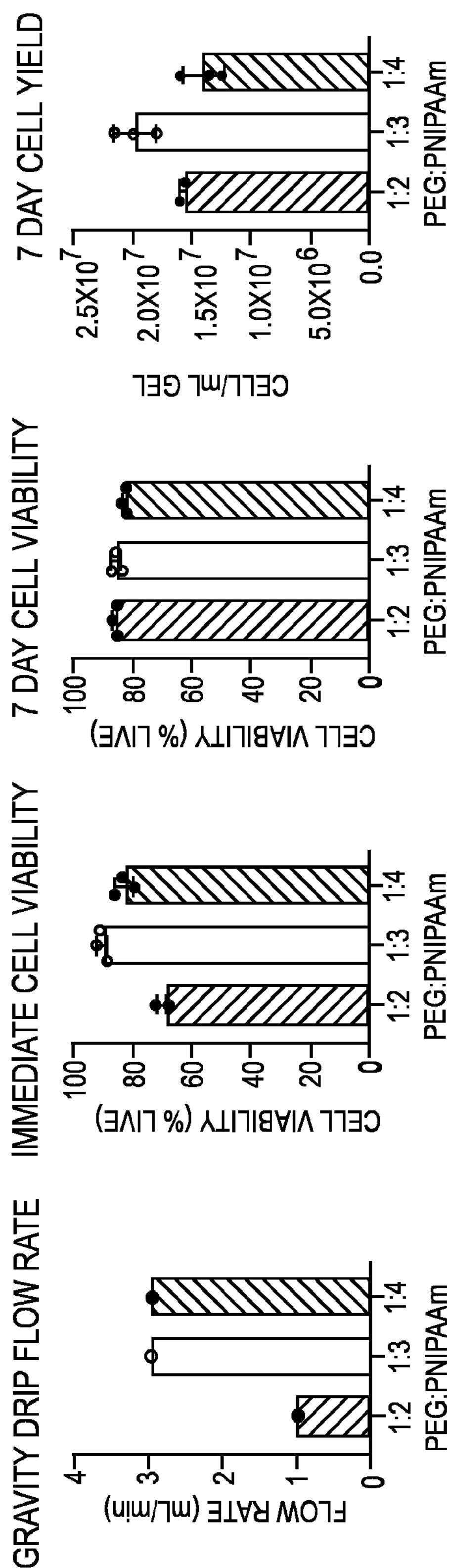


FIG. 5C

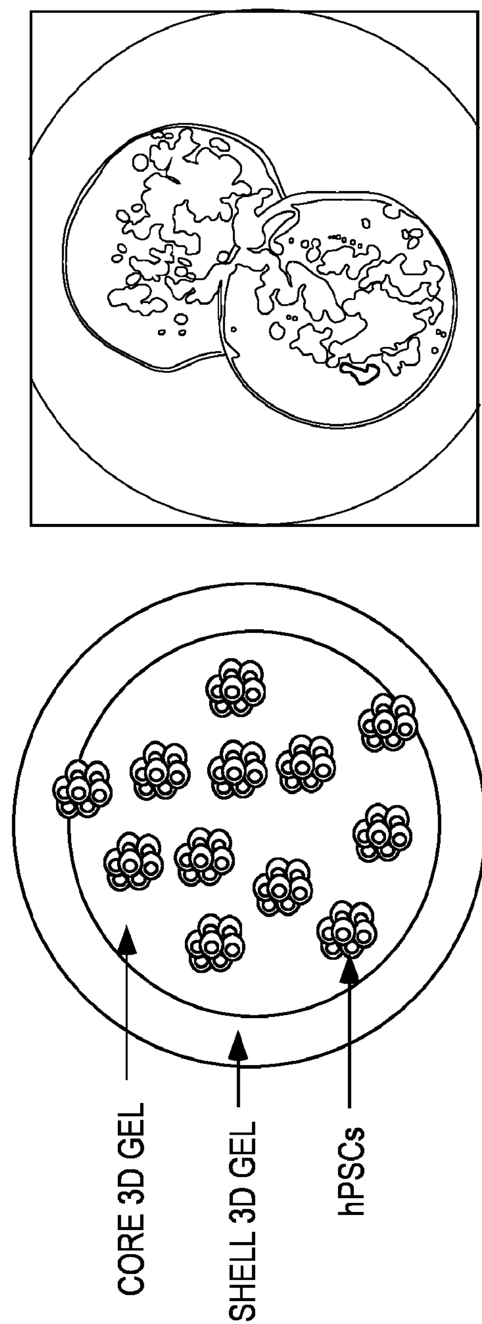


FIG. 5D

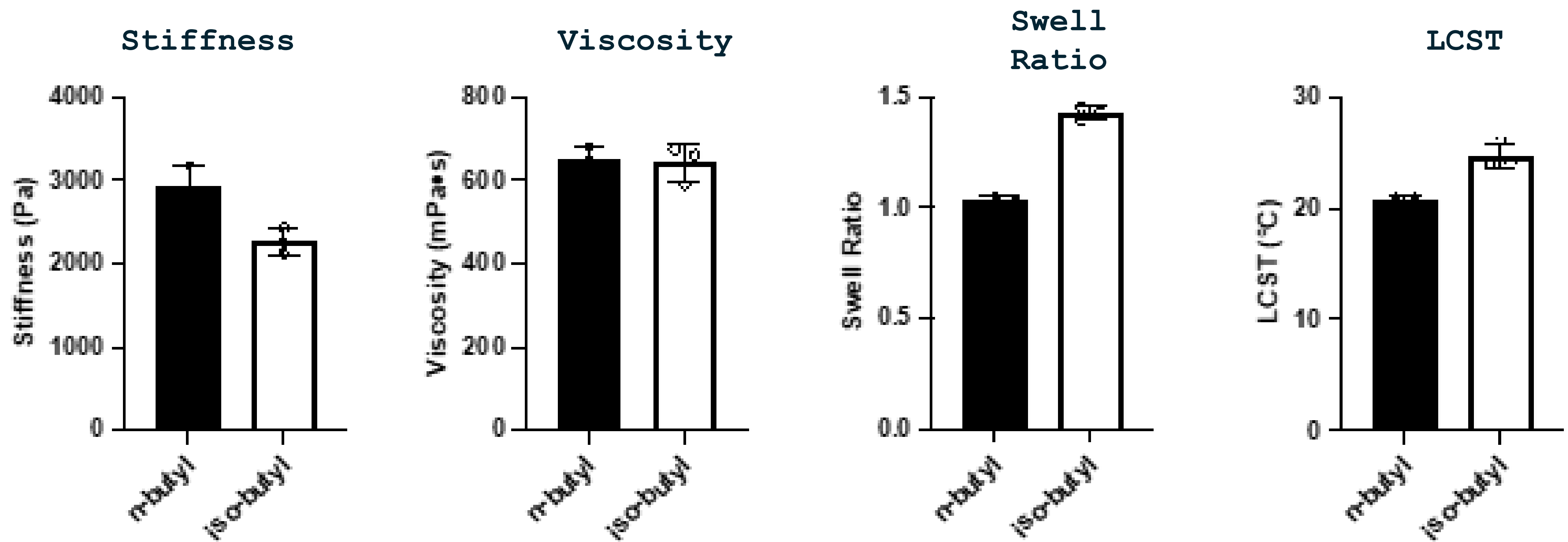


Figure 6A

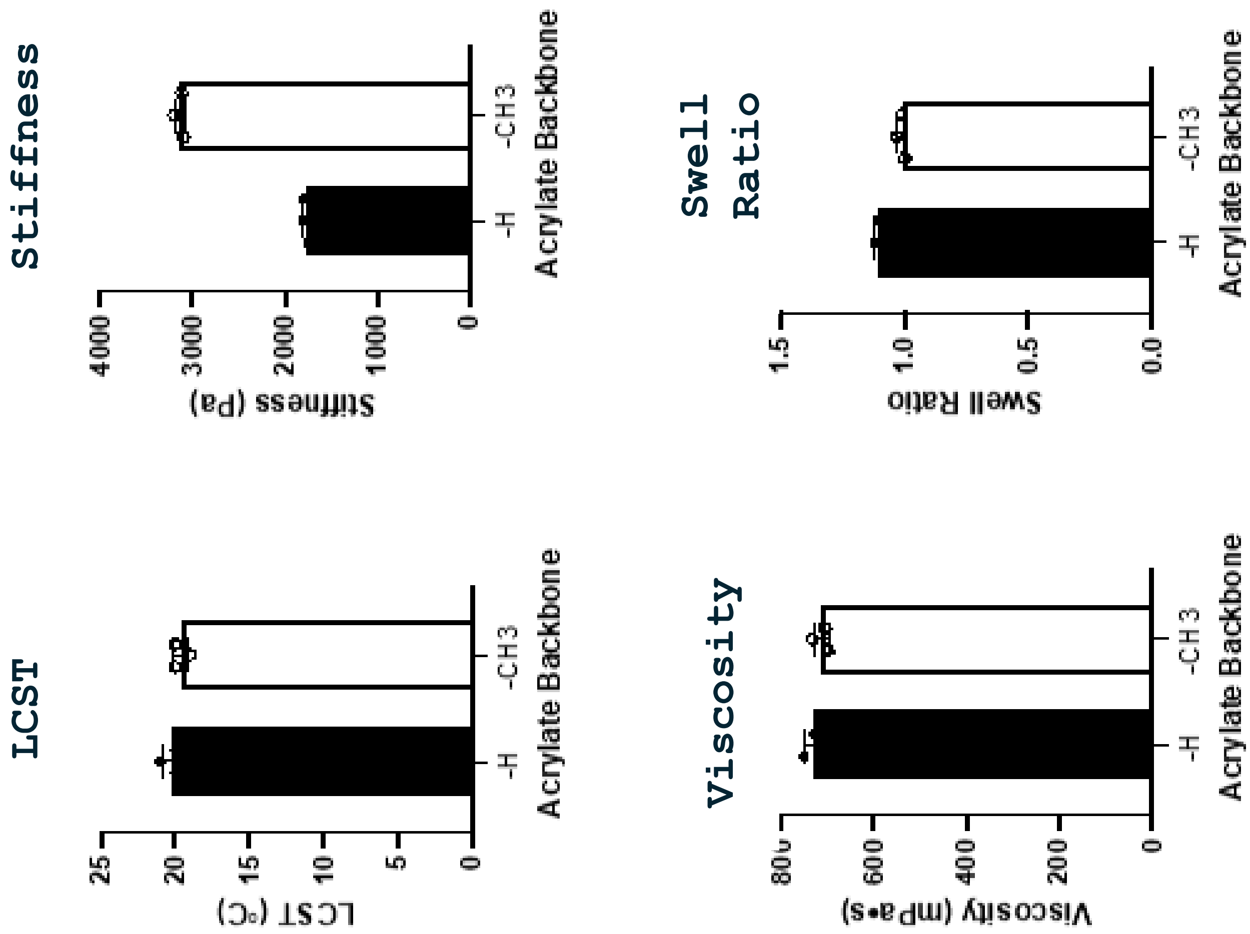
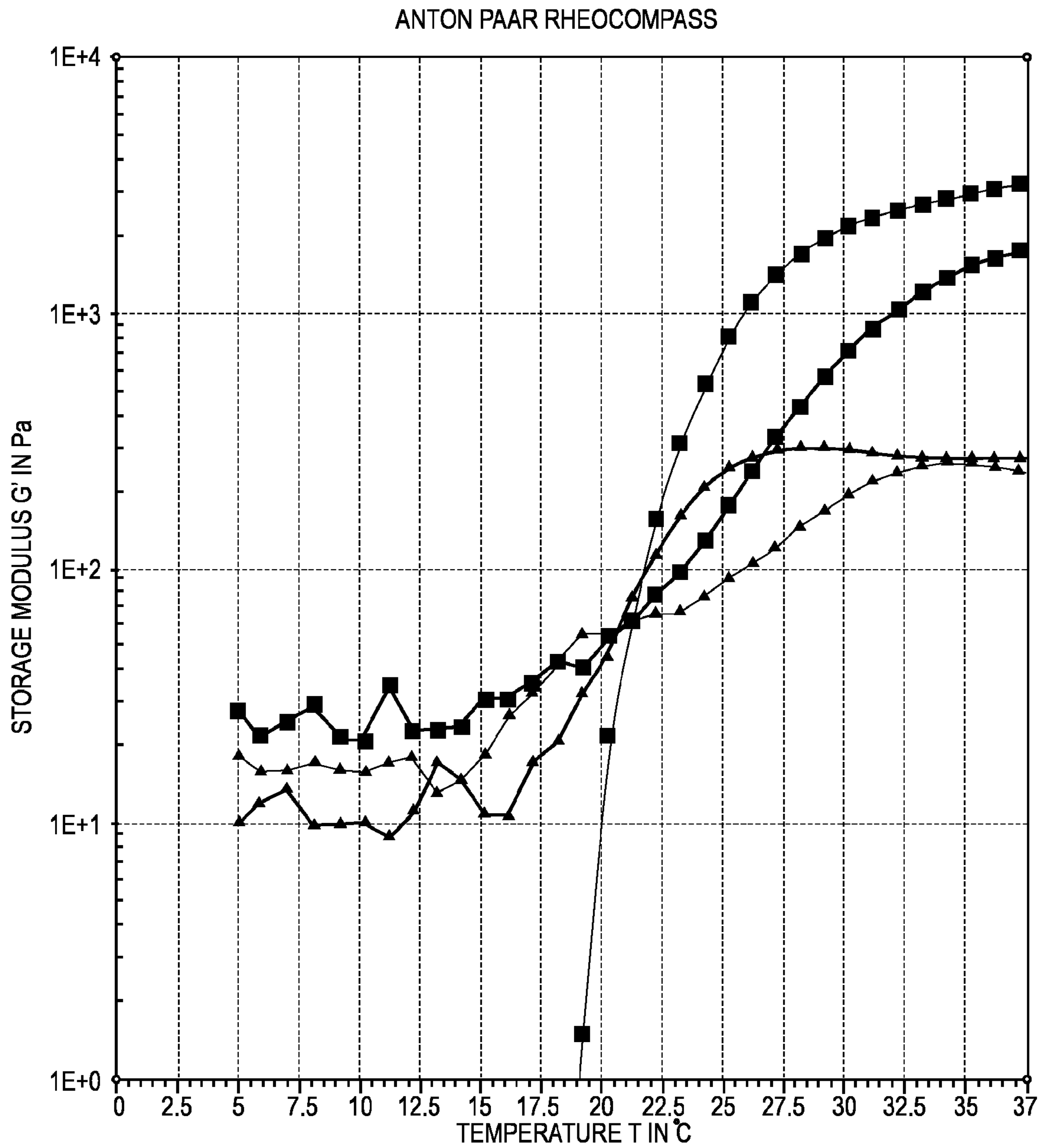


Figure 6B

FIG. 6C



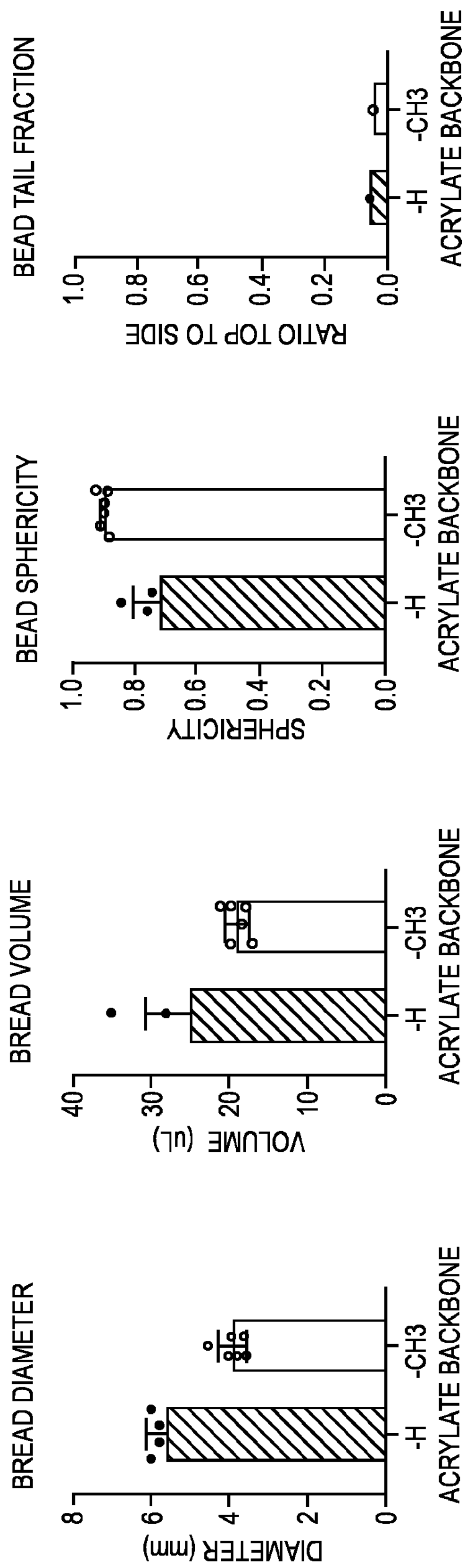
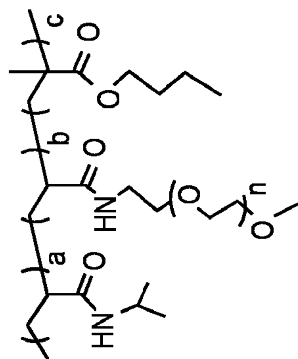
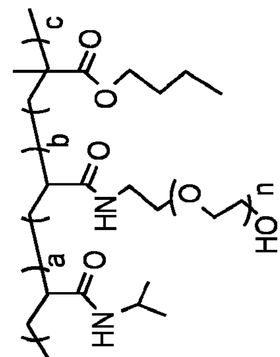


FIG. 6D
BREAD DIAMETER
DIAMETER (mm)
8
6
4
2
0
-H
-CH3
ACRYLATE BACKBONE
BREAD VOLUME
VOLUME (uL)
40
30
20
10
0
-H
-CH3
ACRYLATE BACKBONE
BEAD SPHERICITY
SPHERICITY
1.0
0.8
0.6
0.4
0.2
0.0
-H
-CH3
ACRYLATE BACKBONE
BEAD TAIL FRACTION
RATIO TOP TO SIDE
1.0
0.8
0.6
0.4
0.2
0.0
-H
-CH3
ACRYLATE BACKBONE

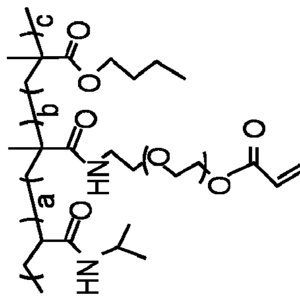
FIG. 7



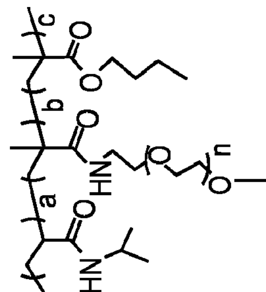
[PNIPAAm-*co*-PBMA]-*b*-[METHOXY-PEG]



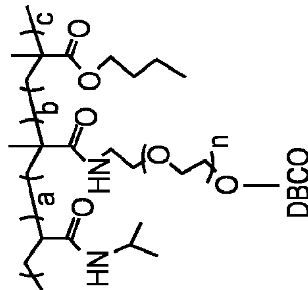
[PNIPAAm-*co*-PBMA]-*b*-[HYDROXYL-PEG]



[PNIPAAm-*co*-PBMA]-*b*-[PEG-ACRYLATE]

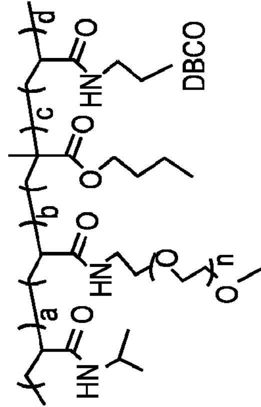


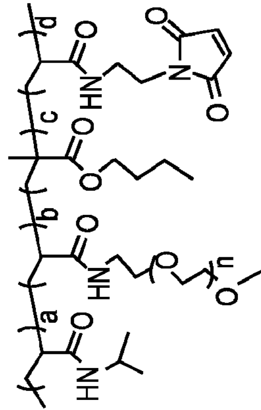
[PNIPAAm-*co*-PBMA]-*b*-[PEG-BIOTIN]

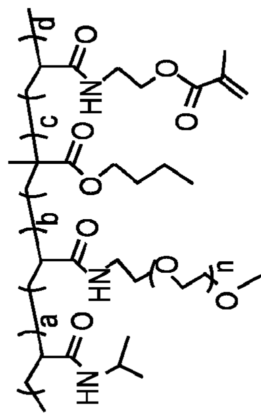


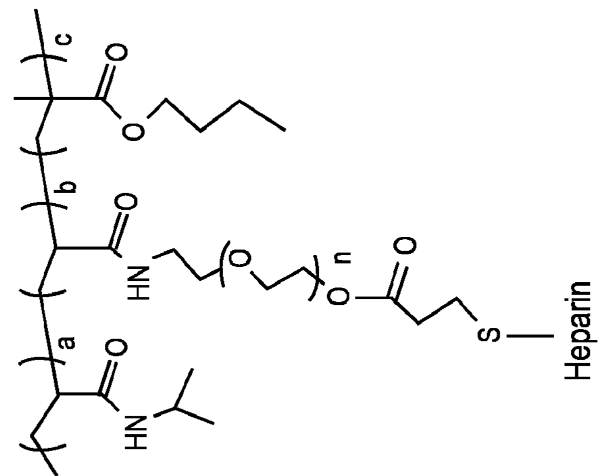
[PNIPAAm-*co*-PBMA]-*b*-[PEG-DBCO]

FIG. 8



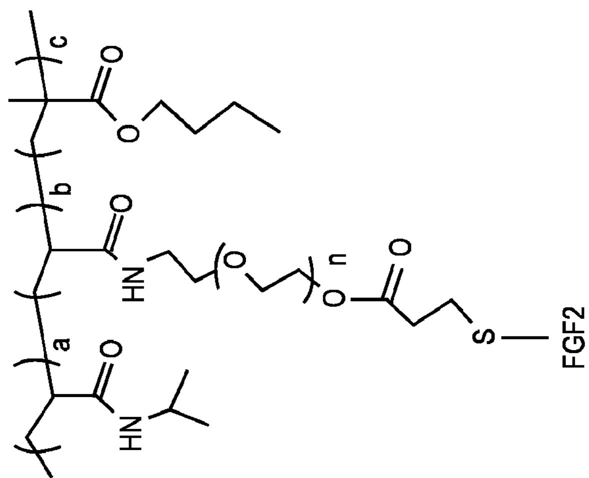






[PNIPAAm-*co*-PBMA]-*b*-[PEG-HEPARIN]

FIG. 9



[PNIPAAm-*co*-PBMA]-*b*-[PEG-FGF]

FIG. 10A

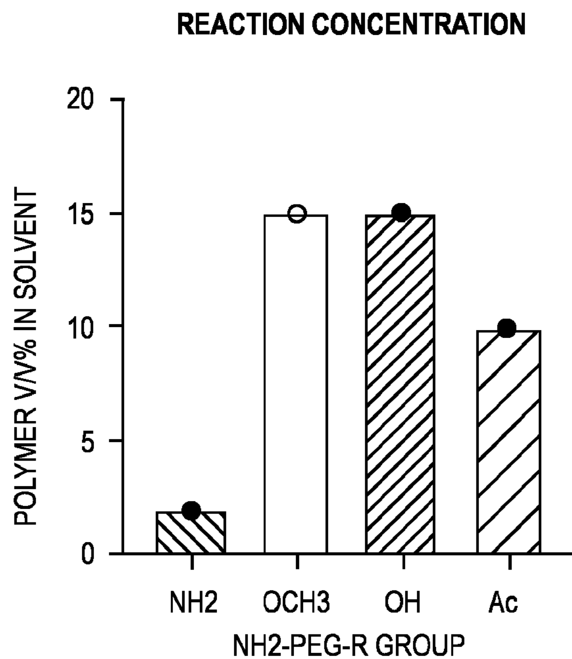
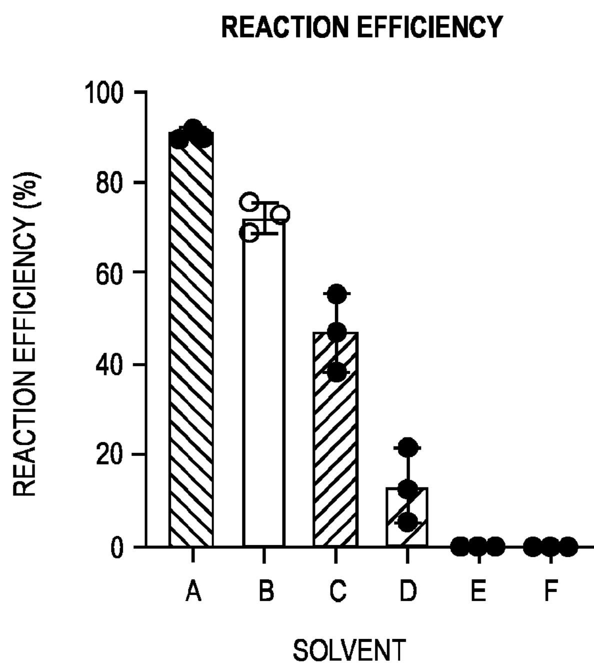


FIG. 10B



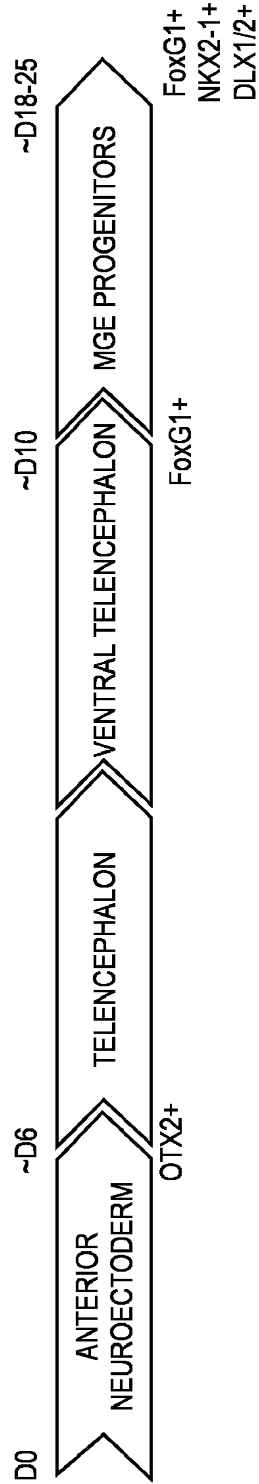


FIG. 11A

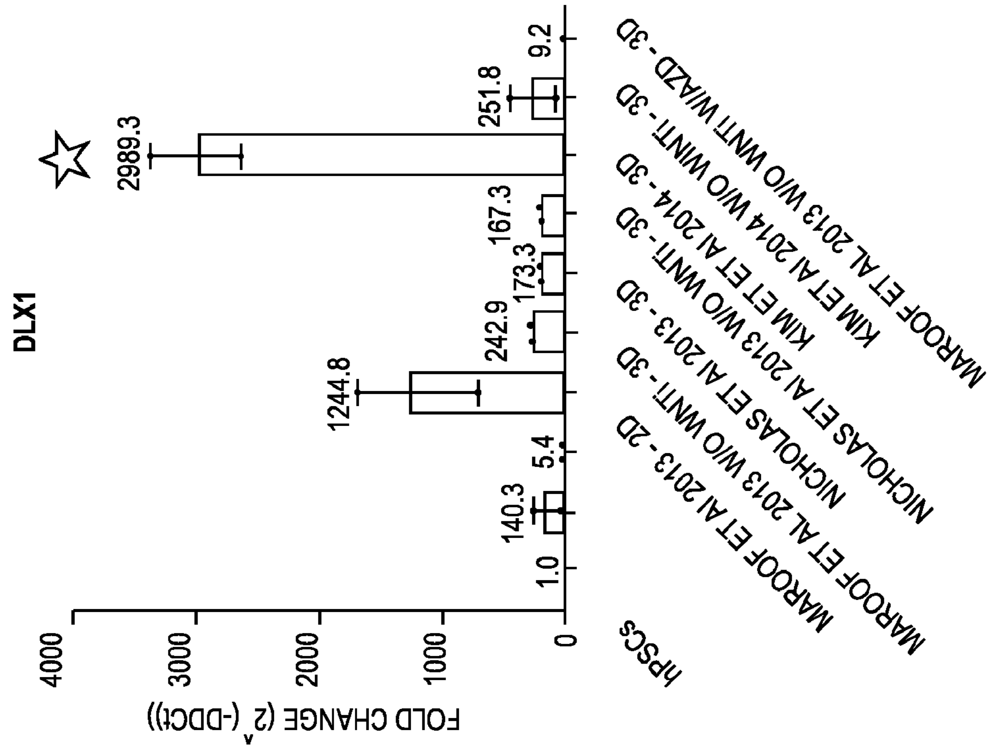


FIG. 11C
DLX1
FOLD CHANGE (2^(-DDCt))
0
1000
2000
3000
4000
1.0
140.3
5.4
1244.8
242.9
173.3
167.3
2989.3
251.8
9.2
hPSCs
MAROOF ET AI 2013 - 2D
MAROOF ET AL 2013 W/O WNTi - 2D
NICHOLAS ET AI 2013 - 3D
NICHOLAS ET AI 2013 W/O WNTi - 3D
KIM ET AI 2014 - 3D
KIM ET AI 2014 W/O WNTi - 3D
MAROOF ET AL 2013 W/O WNTi W/AZD - 3D

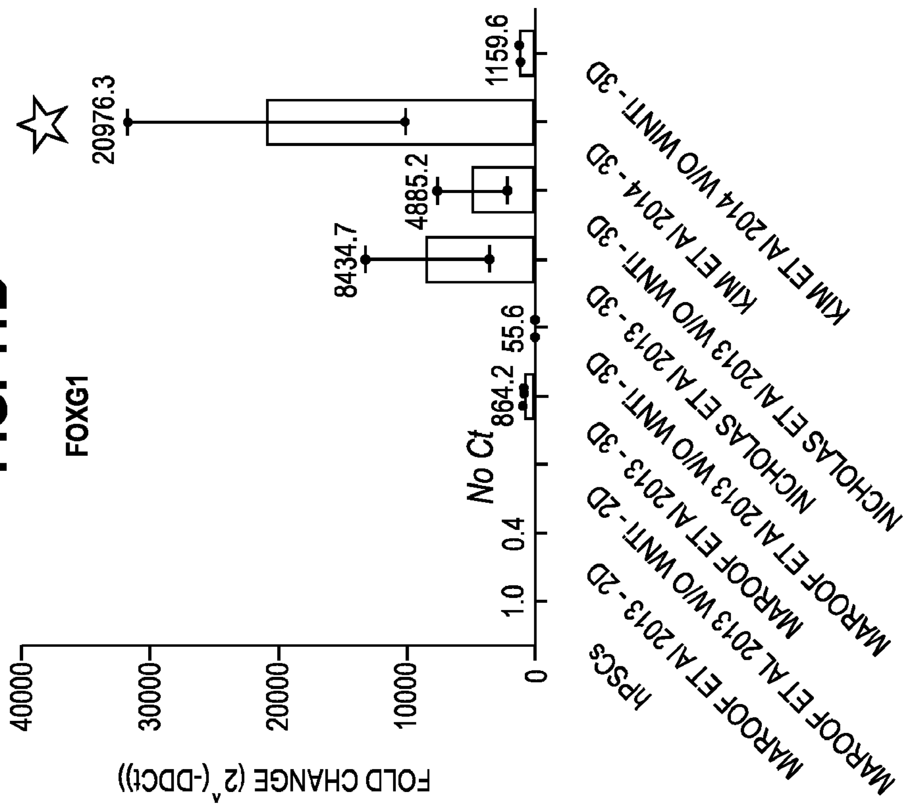


FIG. 11B
FOXG1
FOLD CHANGE (2^(-DDCt))
0
10000
20000
30000
40000
1.0
0.4
No Ct
864.2
55.6
8434.7
4885.2
20976.3
1159.6
hPSCs
MAROOF ET AI 2013 - 2D
MAROOF ET AL 2013 W/O WNTi - 2D
MAROOF ET AI 2013 - 3D
MAROOF ET AI 2013 W/O WNTi - 3D
NICHOLAS ET AI 2013 - 3D
NICHOLAS ET AI 2013 W/O WNTi - 3D
KIM ET AI 2014 - 3D
KIM ET AI 2014 W/O WNTi - 3D

FIG. 11D

FoxG1+Cells (%)

0
20
40
60
80
100

0.55
0.81
58.81
5.48
47.66
43.67
88.42
12.98
0.22

hPSCs
MAROOF ET Al 2013 - 2D
MAROOF ET Al 2013 W/O WNTi - 2D
MAROOF ET AL 2013 - 3D
MAROOF ET Al 2013 W/O WNTi - 3D
NICHOLAS ET Al 2013 - 3D
NICHOLAS ET Al 2013 W/O WNTi - 3D
KIM ET Al 2014 - 3D
KIM ET Al 2014 W/O WINTi - 3D
MAROOF ET AL 2013 W/O WNTI W/AZD - 3D

FIG. 11E

NKx2-1+CELLS (%)

0
20
40
60
80
100

3.90
0.03
28.04
8.29
1.57
0.51
78.38
7.66
0.06

hPSCs
MAROOF ET Al 2013 - 2D
MAROOF ET Al 2013 W/O WNTi - 2D
MAROOF ET AL 2013 - 3D
MAROOF ET Al 2013 W/O WNTi - 3D
NICHOLAS ET Al 2013 - 3D
NICHOLAS ET Al 2013 W/O WNTi - 3D
KIM ET Al 2014 - 3D
KIM ET Al 2014 W/O WINTi - 3D
MAROOF ET AL 2013 W/O WNTI W/AZD - 3D

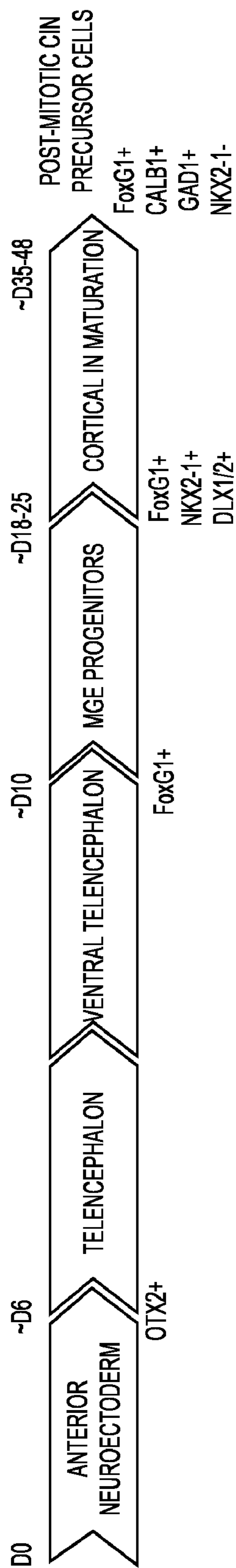


FIG. 12A

FIG. 12B

MGE-Derived Cells

FIG. 12C

INTERNEURON SUBTYPE (MIGRATORY CORTICAL INTERNEURON PRECURORS)

FIG. 12D

GABA BIOSYNTHESIS

GAD1

FOLD CHANGE (2^(-DDCt))

0 20 40 60 80 100

hPSCs: 1.0
MAROOF ET Al 2013 - 2D: 3.7
MAROOF ET AL 2013 W/O WNTi - 2D: 1.5
MAROOF ET Al 2013 - 3D: 12.2
MAROOF ET AL 2013 W/O WNTi - 3D: 20.1
NICHOLAS ET Al 2013 - 3D: 36.2
NICHOLAS ET Al 2013 W/O WNTi - 3D: 10.5
KIM ET Al 2014 - 3D: 83.7
KIM ET Al 2014 W/O WNTi - 3D: 43.2
MAROOF ET AL 2013 W/O WNTi W/AZD - 3D: 1.2

FIG. 12E

FoxG1+Cells (%)

0 20 40 60 80 100

MAROOF ET Al 2013 - 2D: 0.46
MAROOF ET AL 2013 W/O WNTi - 2D: 0.12
MAROOF ET Al 2013 - 3D: 43.24
MAROOF ET AL 2013 W/O WNTi - 3D: 17.85
NICHOLAS ET Al 2013 - 3D: 14.60
NICHOLAS ET Al 2013 W/O WNTi - 3D: 23.53
KIM ET Al 2014 - 3D: 78.14
KIM ET Al 2014 W/O WNTi - 3D: 22.97
MAROOF ET AL 2013 W/O WNTi W/AZD - 3D: 0.11

FIG. 12F

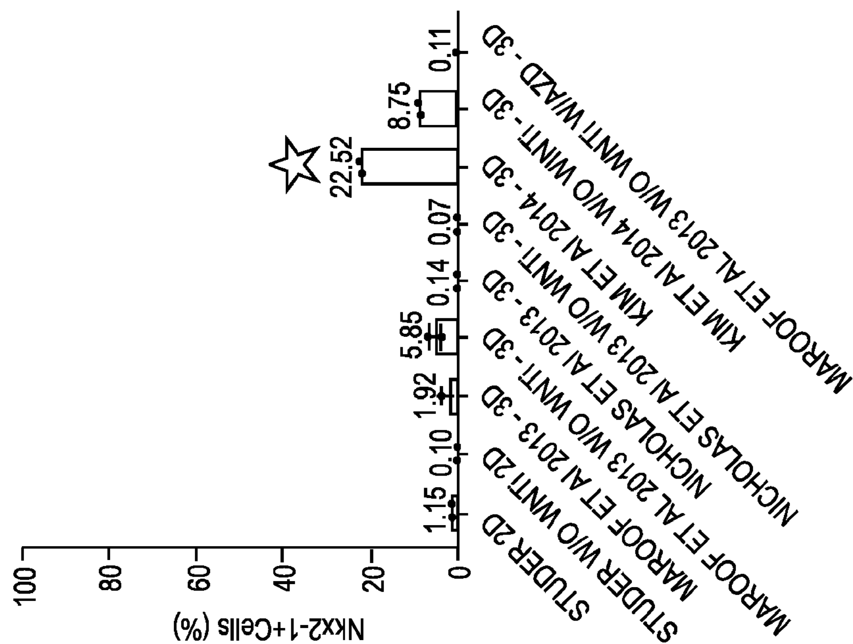
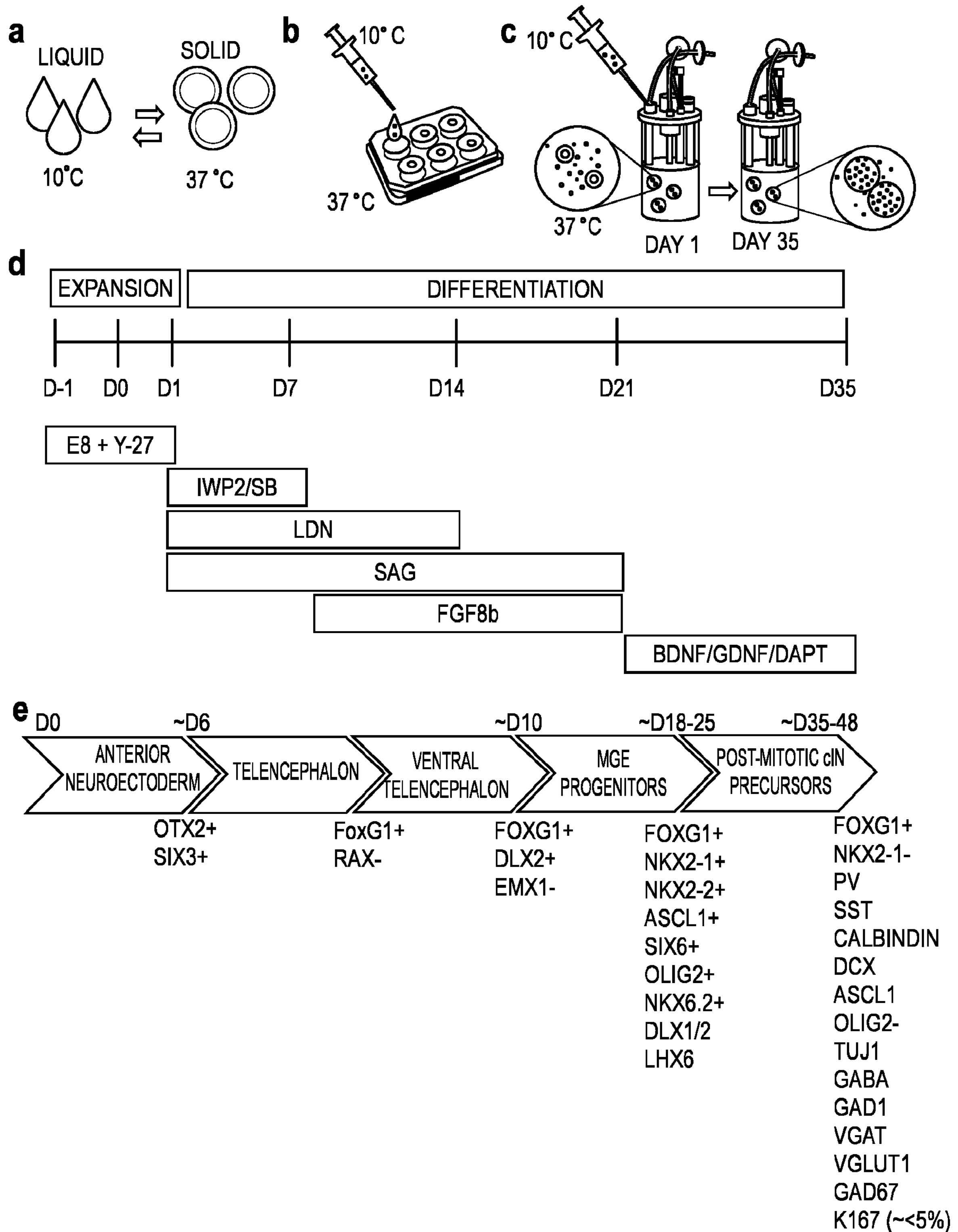


FIG. 13



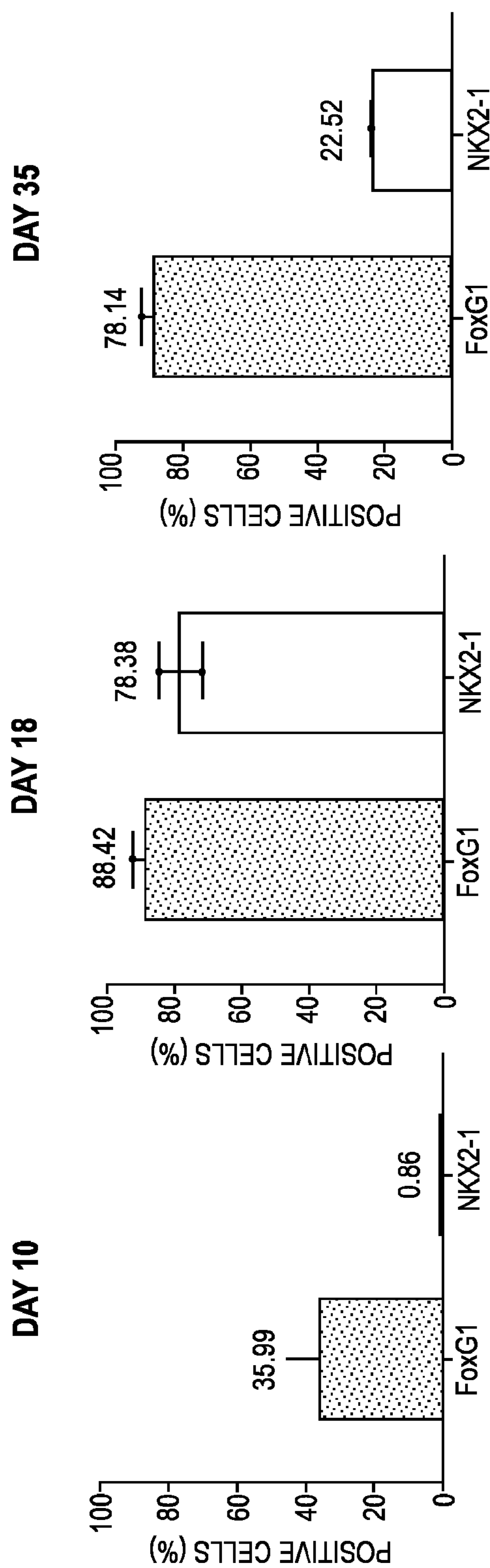


FIG. 14A

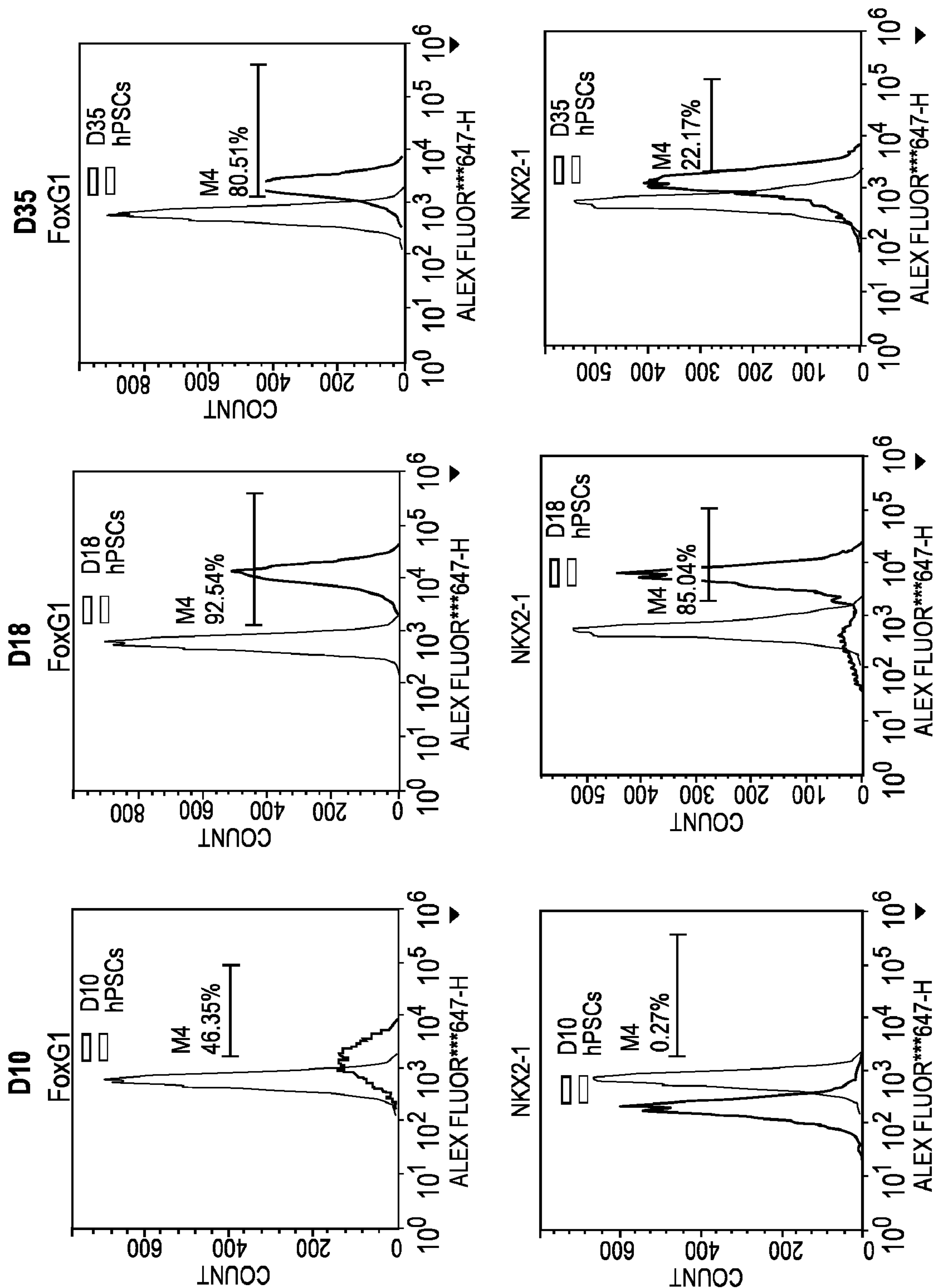


FIG. 14B
D10
FoxG1
D10
hPSCs
M4
46.35%
COUNT
ALEX FLUOR***647-H
D18
FoxG1
D18
hPSCs
M4
92.54%
COUNT
ALEX FLUOR***647-H
D35
FoxG1
D35
hPSCs
M4
80.51%
COUNT
ALEX FLUOR***647-H
NKX2-1
D10
hPSCs
M4
0.27%
COUNT
ALEX FLUOR***647-H
NKX2-1
D18
hPSCs
M4
85.04%
COUNT
ALEX FLUOR***647-H
NKX2-1
D35
hPSCs
M4
22.17%
COUNT
ALEX FLUOR***647-H

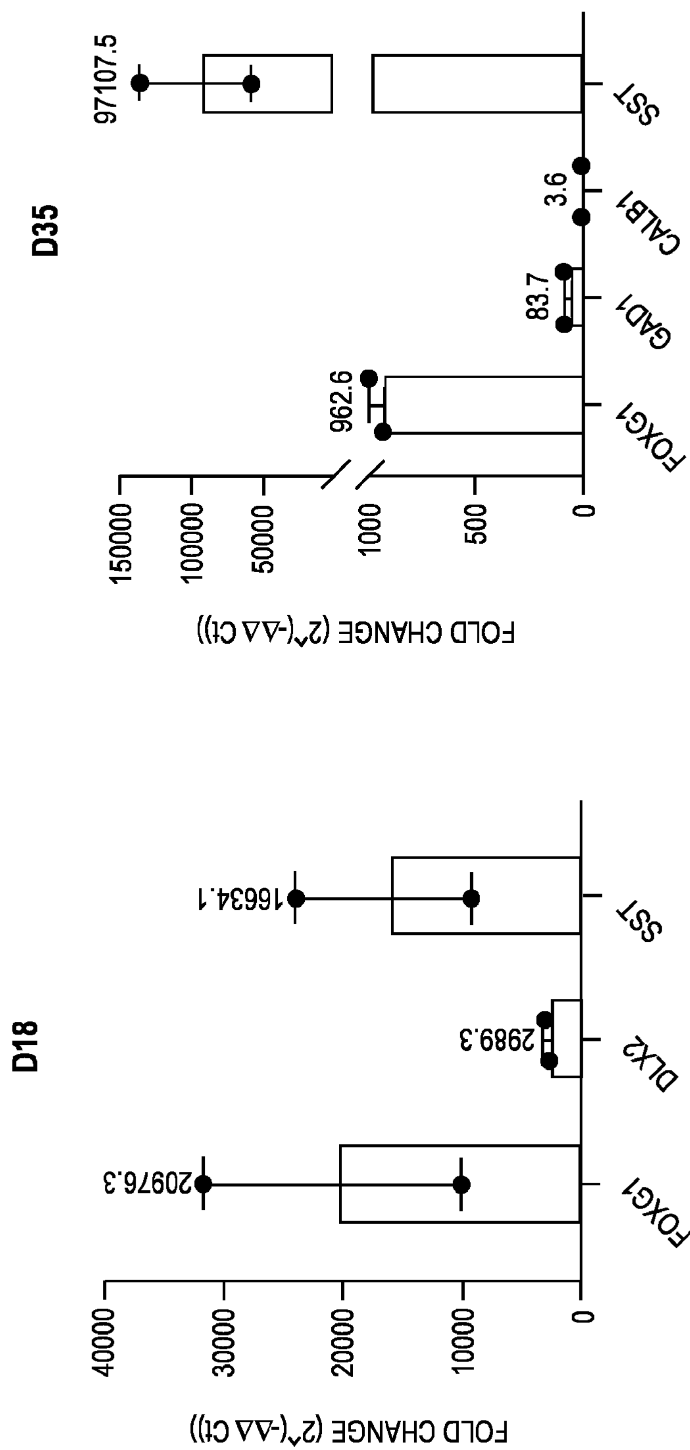


FIG. 15A

FIG. 15B

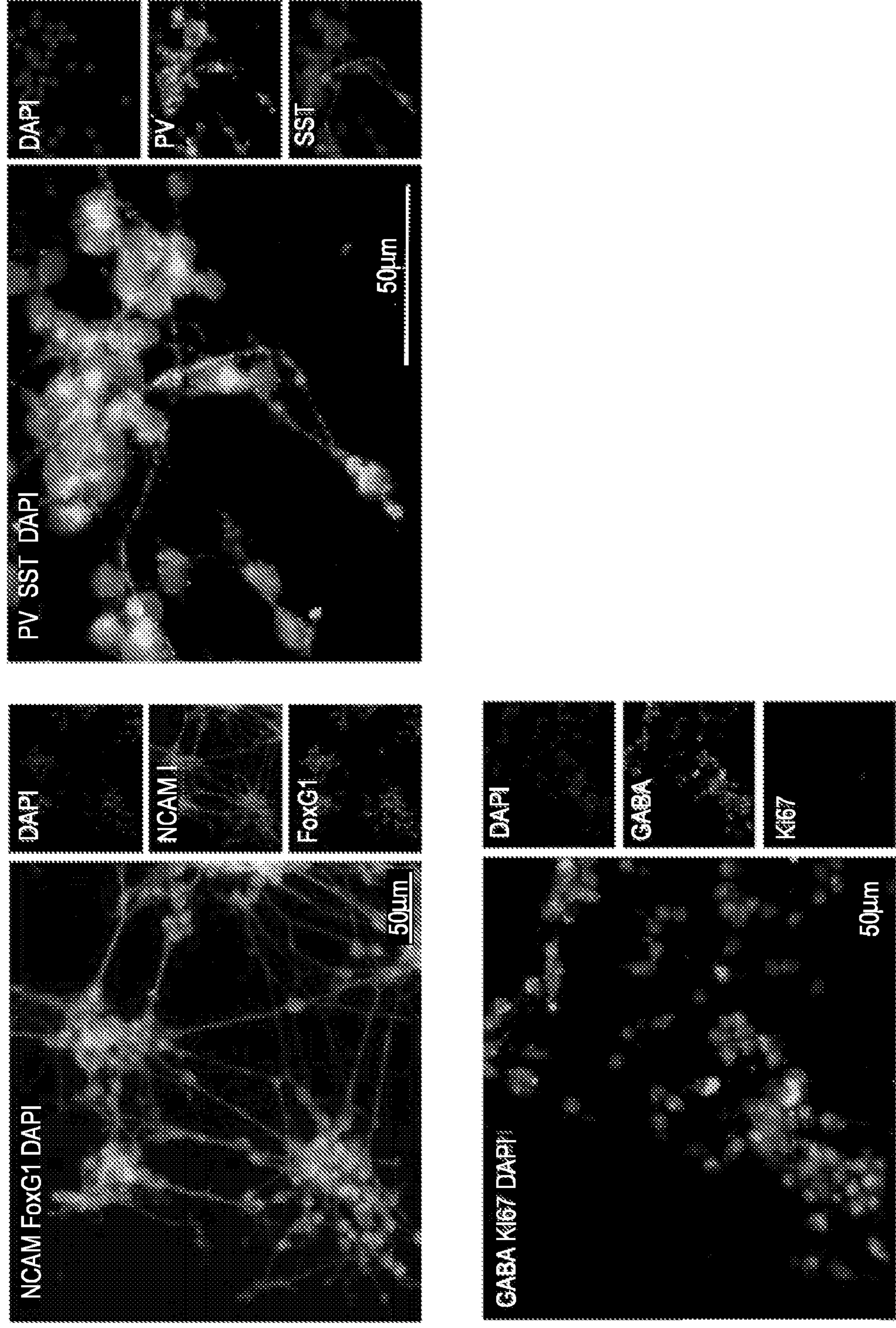


FIG. 15C

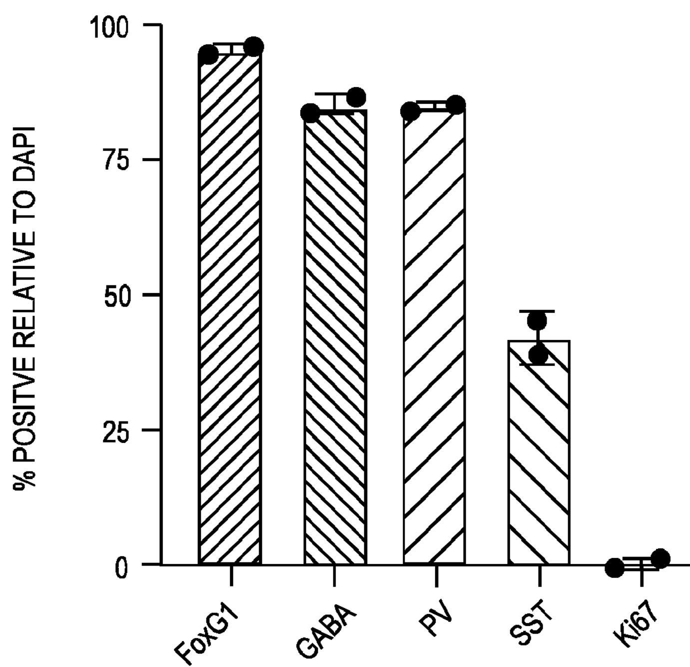
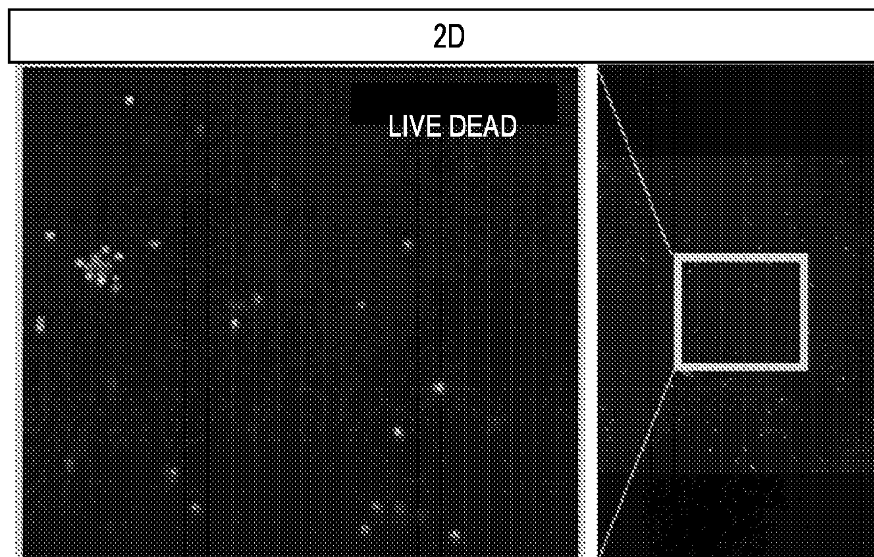


FIG. 16A



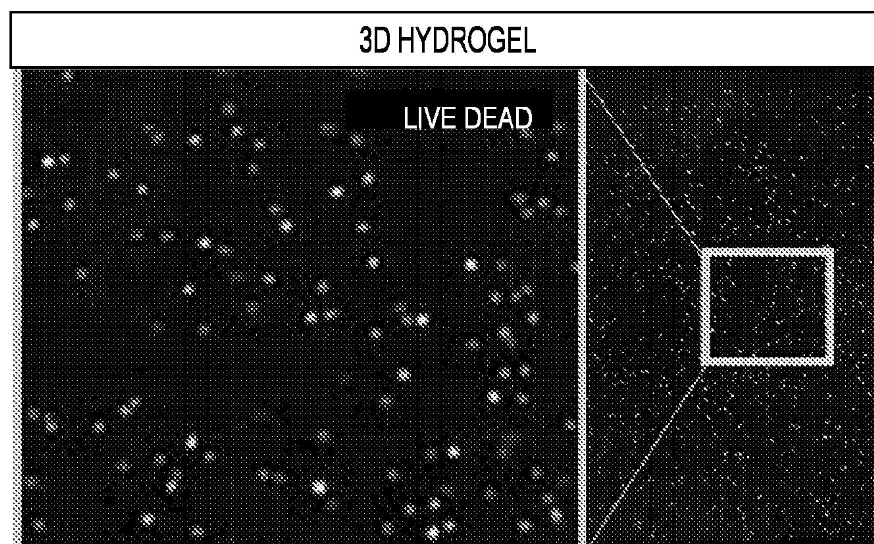
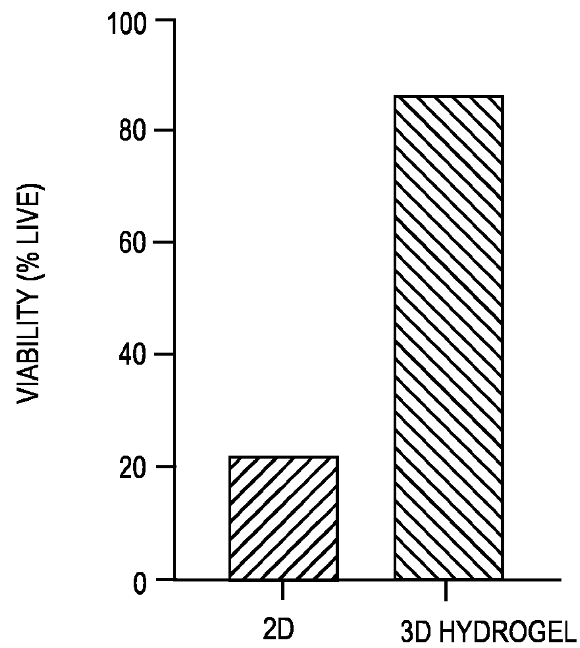


FIG. 16B

FIG. 16B



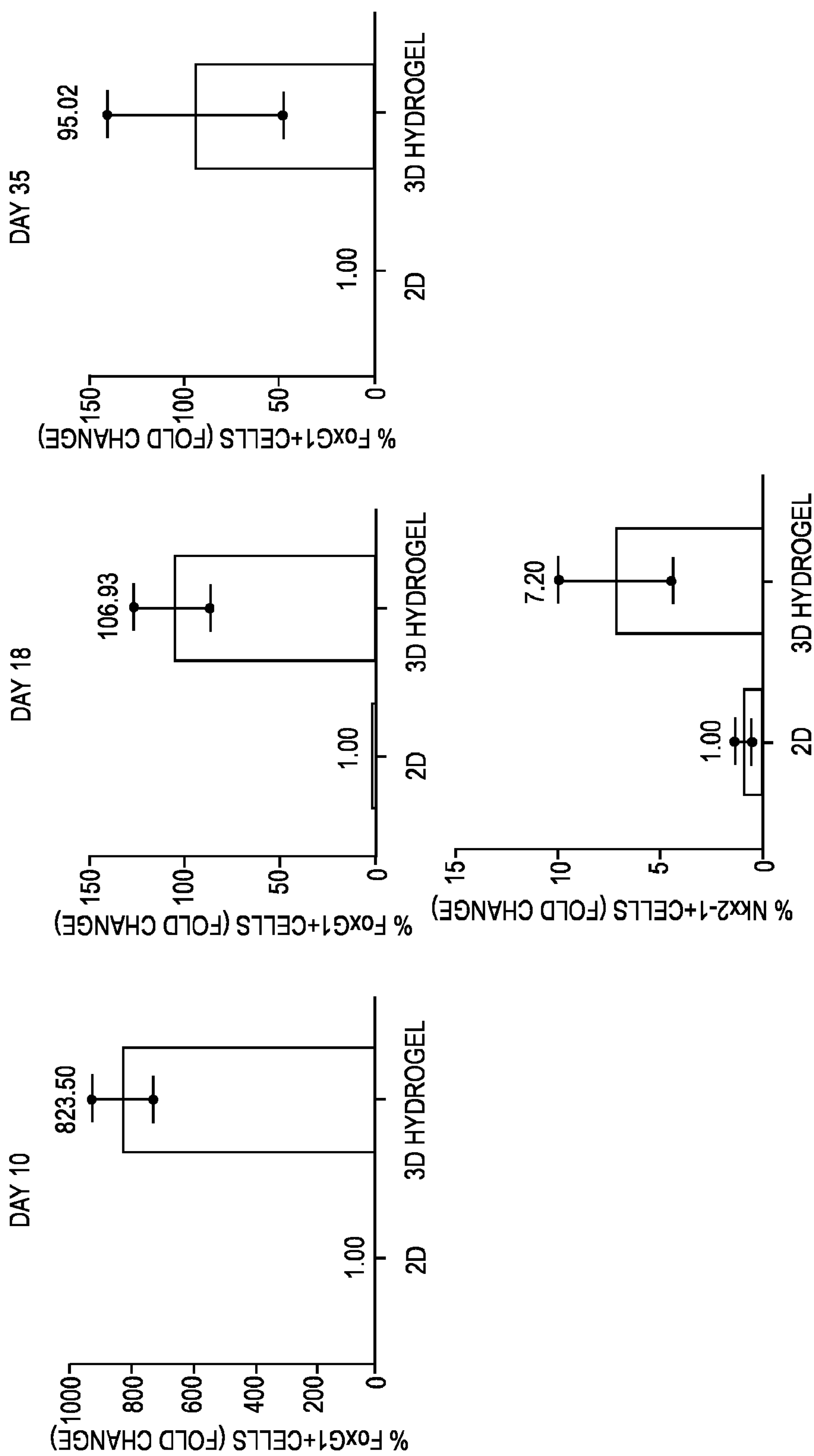


FIG. 16C
DAY 10
% FoxG1+CELLS (FOLD CHANGE)
1000
800
600
400
200
0
1.00
823.50
2D
3D HYDROGEL
DAY 18
% FoxG1+CELLS (FOLD CHANGE)
150
100
50
0
1.00
106.93
2D
3D HYDROGEL
DAY 35
% FoxG1+CELLS (FOLD CHANGE)
150
100
50
0
1.00
95.02
2D
3D HYDROGEL
% Nkx2-1+CELLS (FOLD CHANGE)
15
10
5
0
1.00
7.20
2D
3D HYDROGEL

FIG. 17A

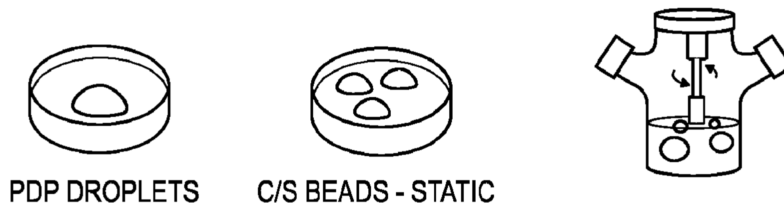


FIG. 17B

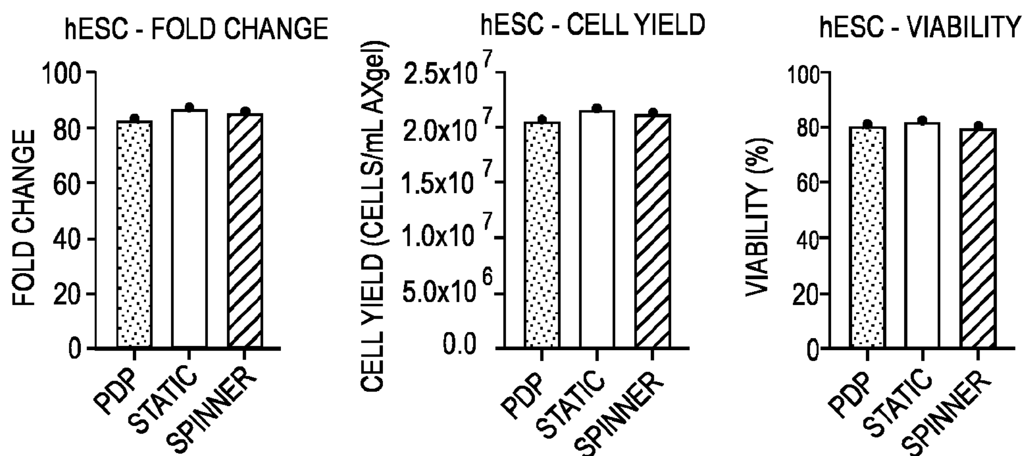


FIG. 17C

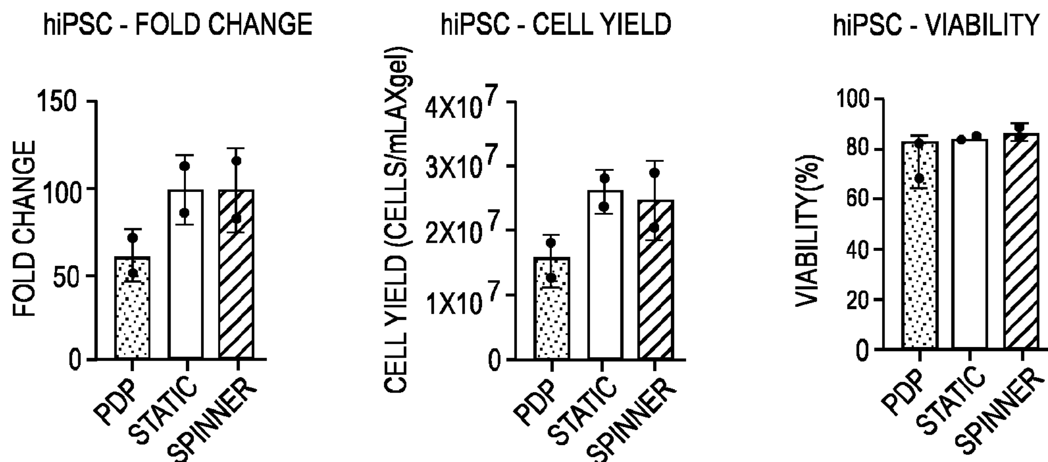
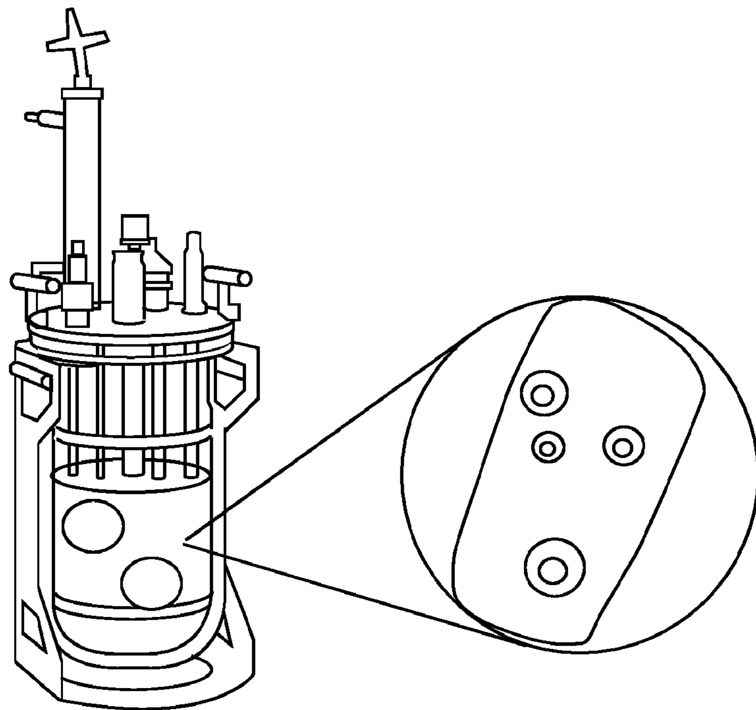


FIG. 18A



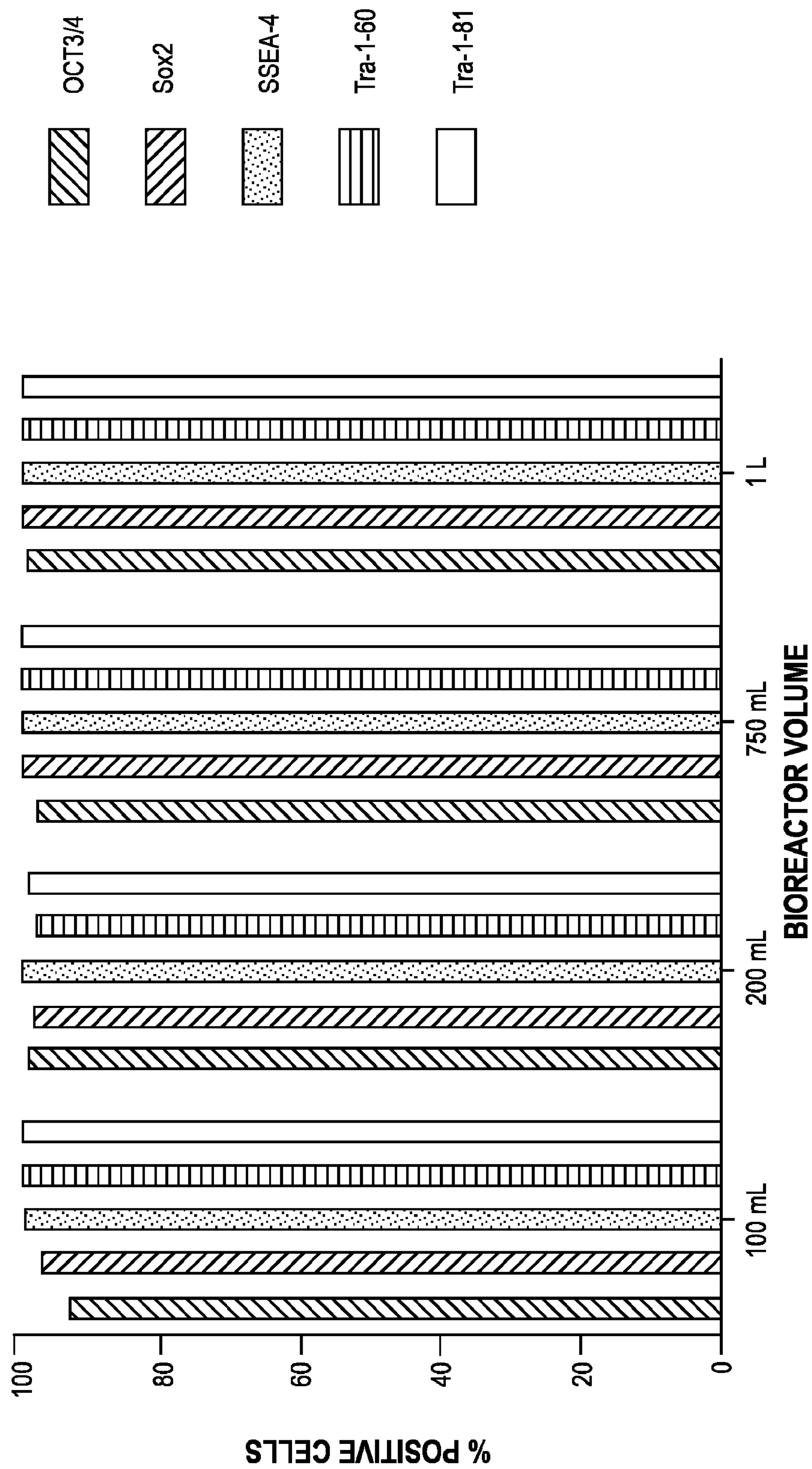
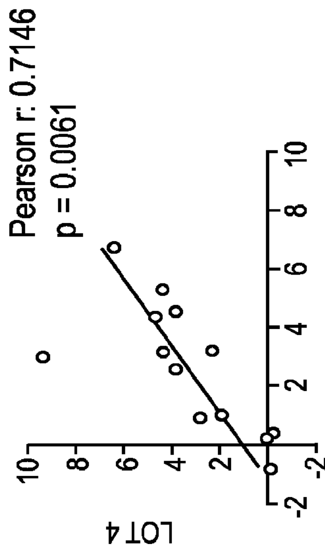
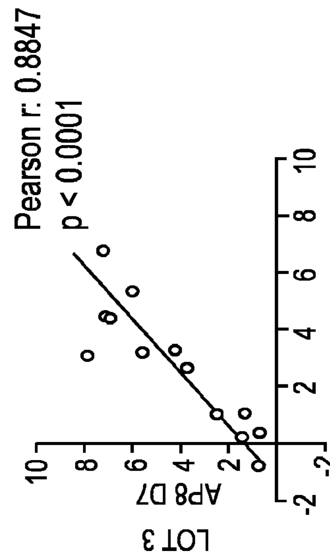
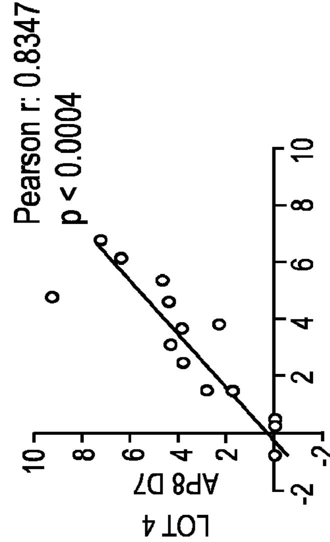


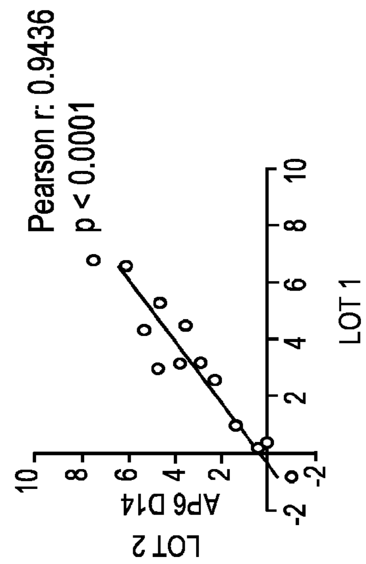
FIG. 18B

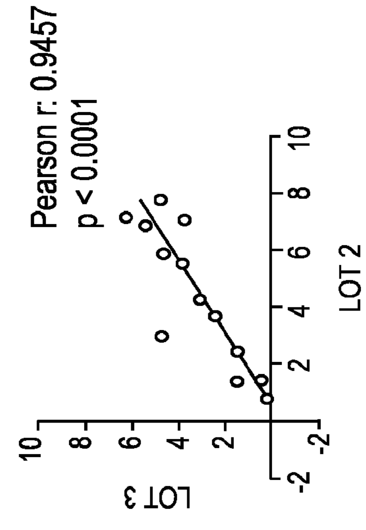
FIG. 18C











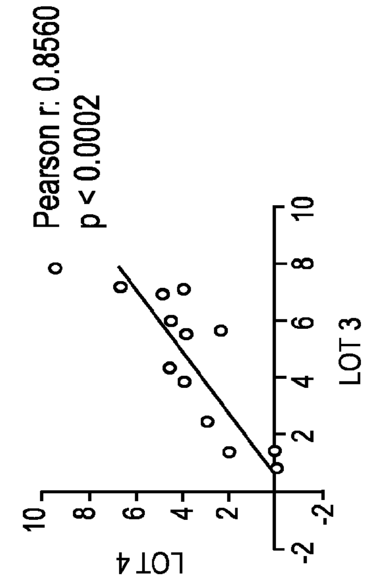


FIG. 19

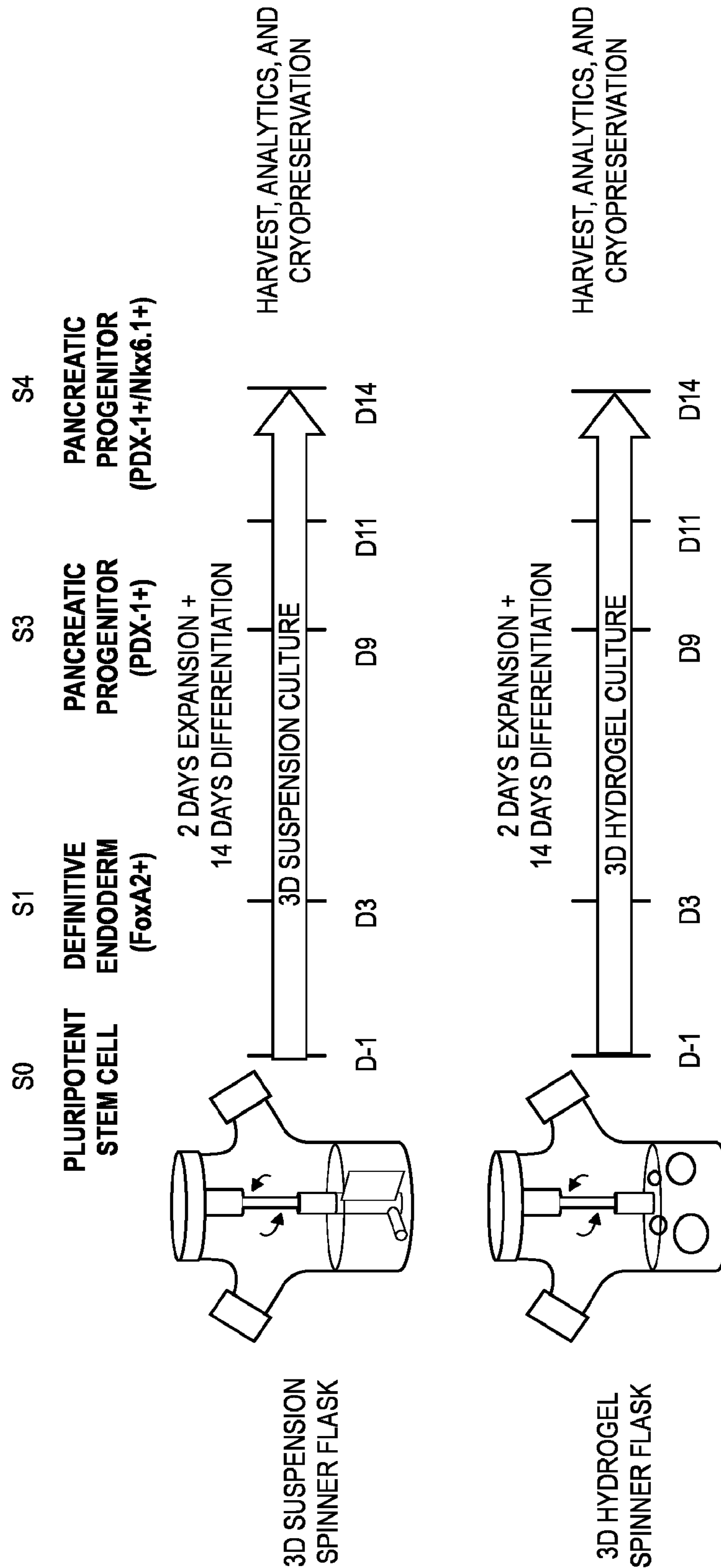


FIG. 20A

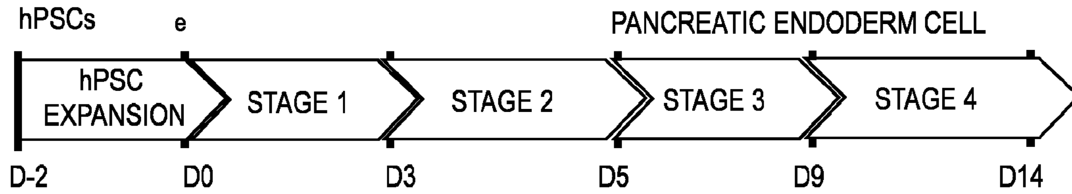


FIG. 20B

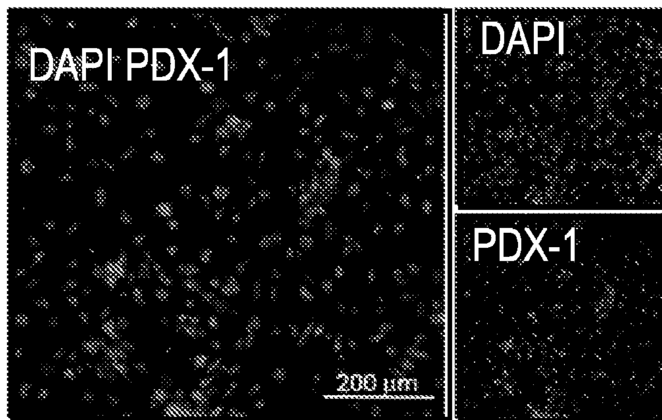


FIG. 20C

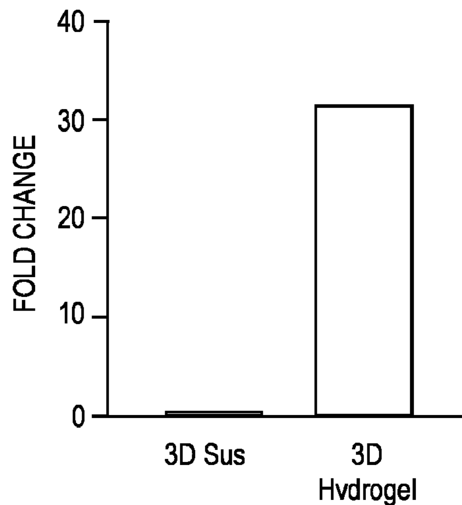


FIG. 20D

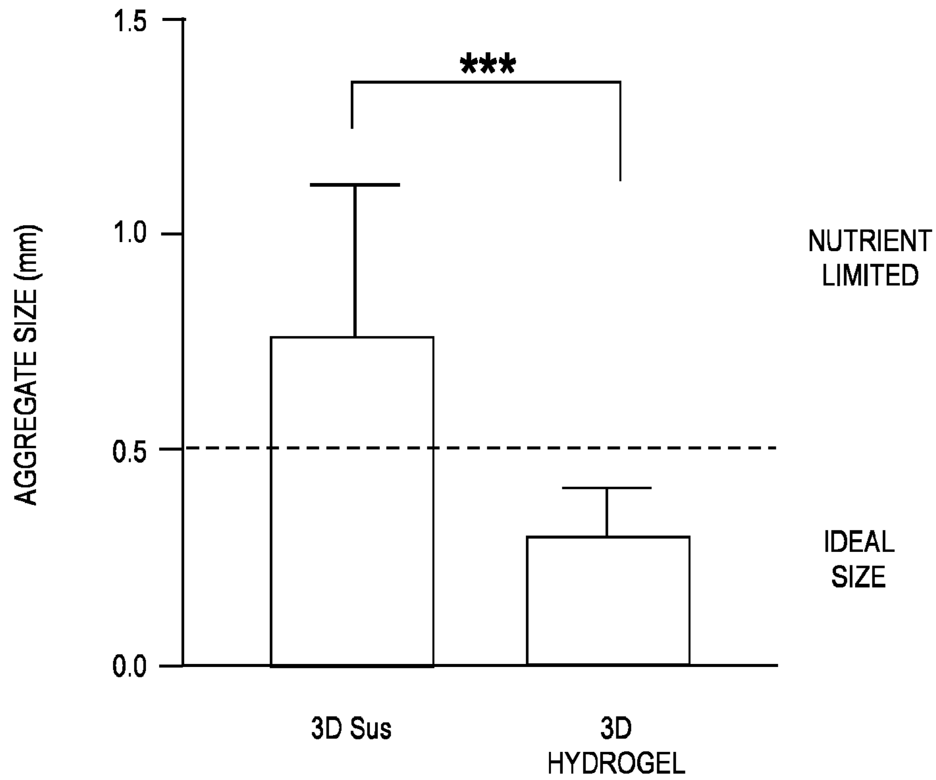


FIG. 21A

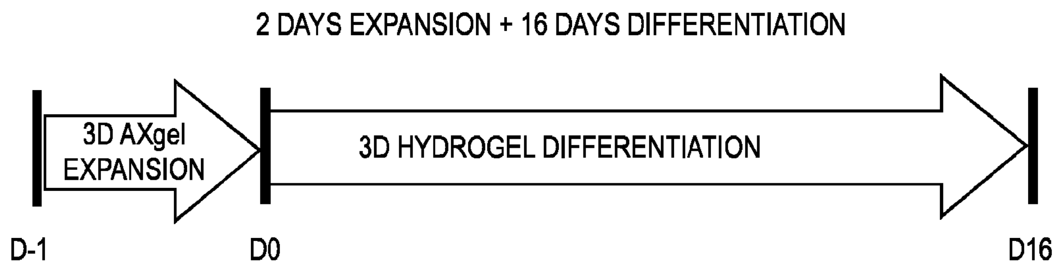


FIG. 21B

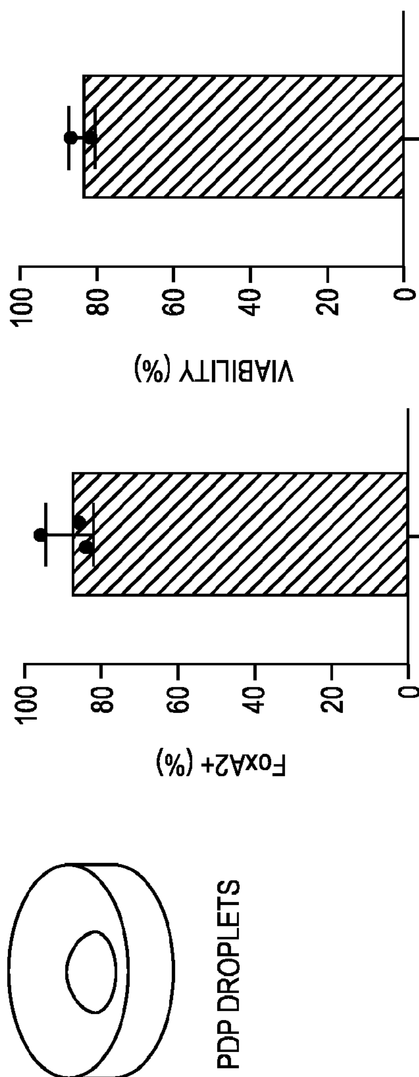
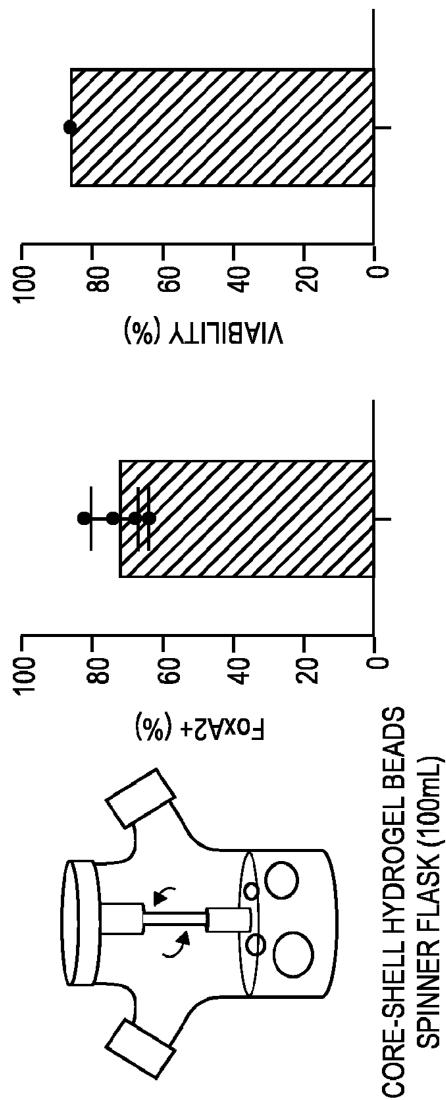


FIG. 21C



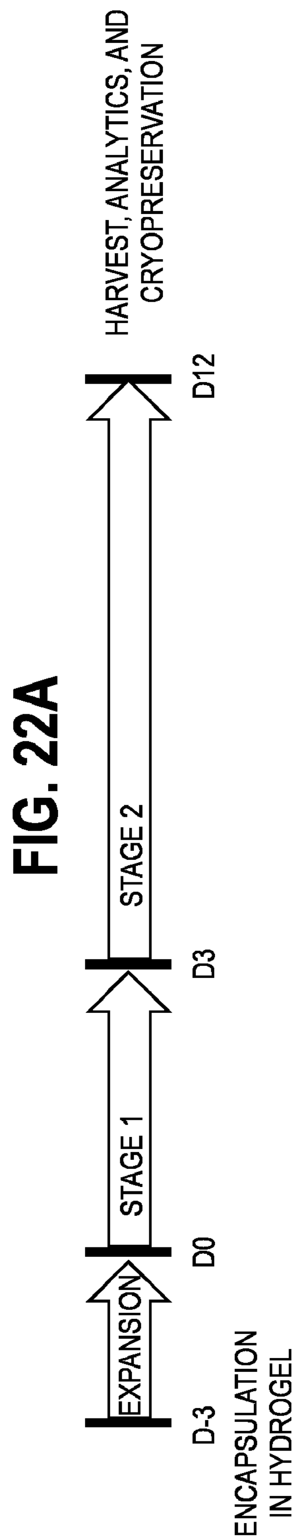


FIG. 22A

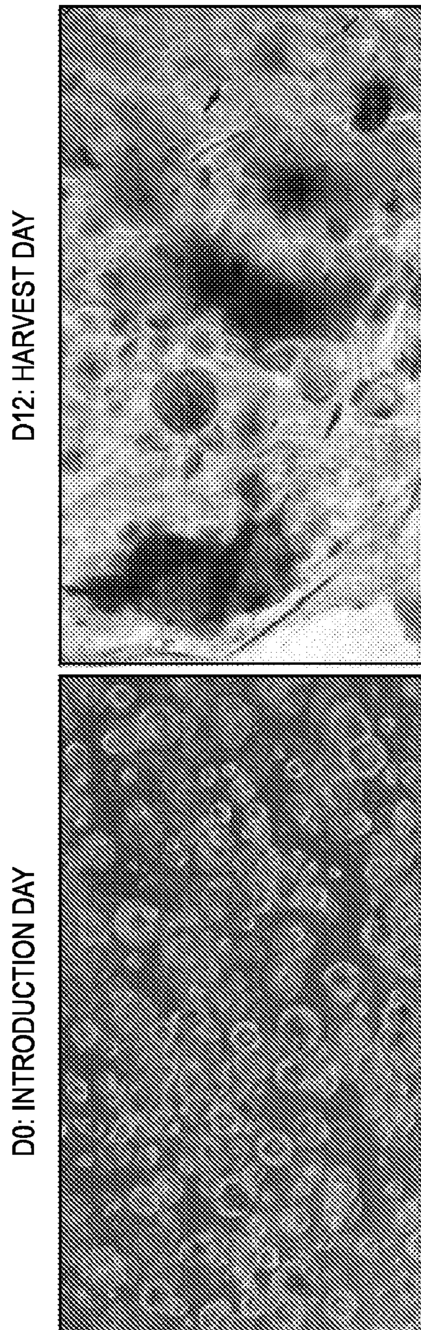


FIG. 22B

FIG. 22C

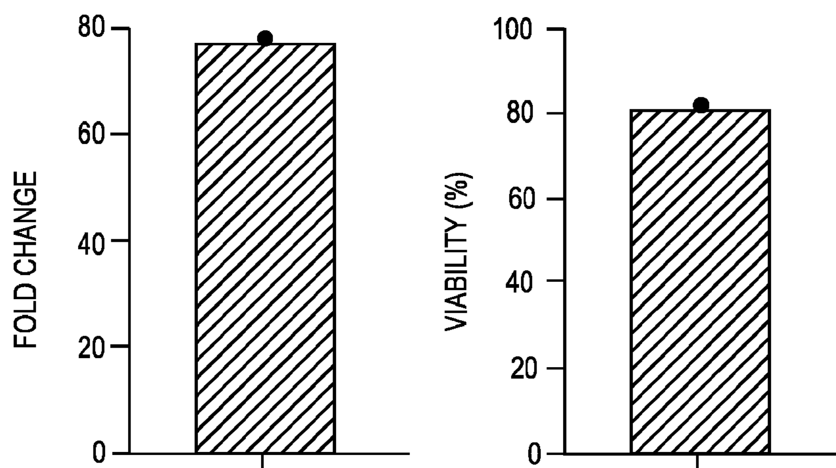


FIG. 23A

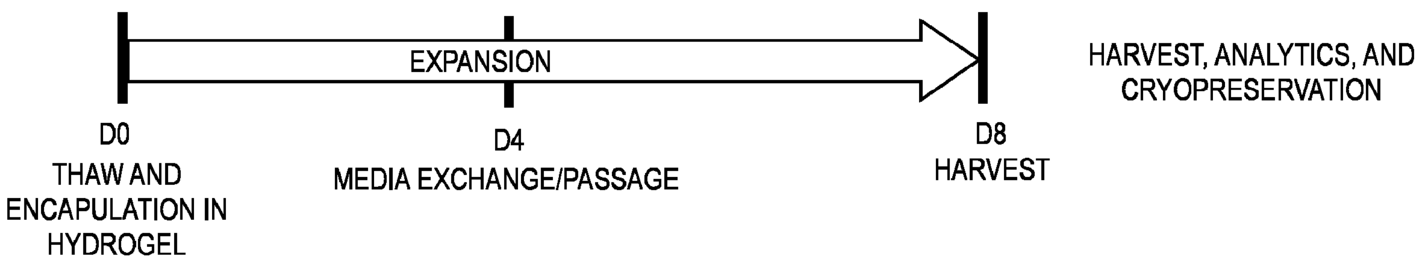


FIG. 23B

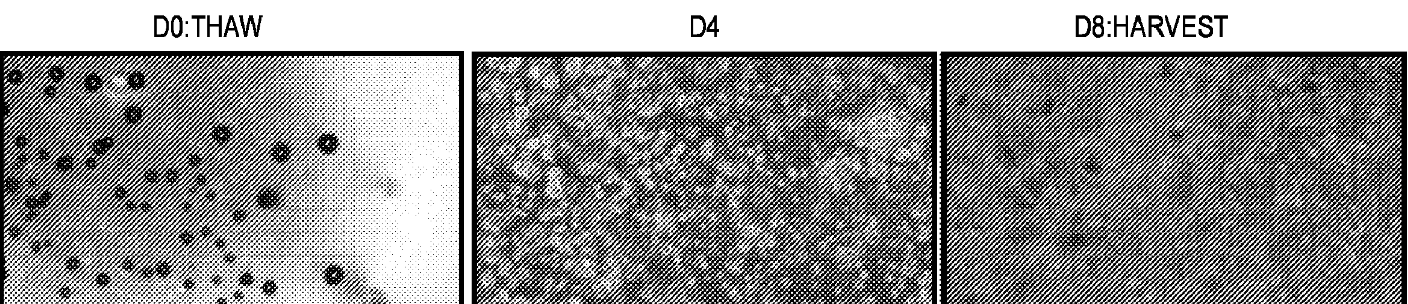


FIG. 23C