3DCellMakers: Thermogelling Polymers for 3D Cell Culture

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SUPPLEMENTAL DATA

General Cell-growth Method using 3DCellMaker

- Hydrogels were weighed into scintillation vials and sterilized using Anprolene AN74i ethylene oxide gas sterilizer for 12 hour cycle per manufacturer's instructions.
- Hydrogels were then dissolved in cell growth media which consisted of DMEM/F12 + Glutamax™ basal media supplemented with 5% (v/v) fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 mg/mL).
- Appropriate amount of growth media was added to give desired % (w/v) concentration for each hydrogel. After adding cell growth media hydrogels were allowed to fully dissolve at refrigerated conditions (approximately 2-8 ^{o}C) for ~48 hours.
- Generally, A3DH (warm use) prepared at 5% (w/v).
- Generally, A3DC (cold use) prepared at 10% (w/v).
- Slightly lower % (w/v) can be used for specific applications.
- MCF-7 breast cancer cells were cultured in 75 cm^2 flasks until ~70-90% confluence was reached.
- Cells were then trypsinized and centrifuged to obtain a cell pellet.
- Cell pellet was re-suspended in fresh media or in cold hydrogels dissolved in media.
- Mixture with cold hydrogel - the hydrogel/cell mixture was transferred into flat bottom polystyrene multiwall plate (un-coated) and incubated 37 °C/ 5% CO_2.
- Pre-warmed hydrogel – media/gel mixture was pre-warmed (incubated 37 °C/ 5% CO_2 for 60-90 minutes in polystyrene multiwall plates and cell suspension was pipetted on top of the solidified gel.
- Both were used to see if there were differences between loading technique for cell growth pattern and morphology.
- At predetermined incubation time points ranging from 24 hours to 7 days the plates were pulled from the incubator and imaged. During the incubation time, media was replaced approximately every 48-72 hours or as color change of media indicated a drop in pH.
- All growth was monitored using AMScope IN300T-FL inverted microscope and photos were taken using AMScope MT5000 camera with IS Capture microscope imaging software.
- Testing of CCD-1068SK fibroblast cells, HEP G2 hepatic cancer cells, and Vero African green monkey kidney cells were conducted in a similar manner as MCF-7 cells.
- Initial growth media used consisted of Minimum Essential Medium Eagle basal media supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 µg/mL).
- Growth media was then transitioned to the same as for MCF-7 but with an increased fetal bovine serum concentration (10% v/v). This was done to utilize the same media for both cell types in anticipation of co-culture experiments.
- Eventually all cell lines were grown in complete growth media which consisted of Minimum Essential Medium Eagle basal media supplemented with 2 mM L-glutamine, 8% (v/v) fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 µg/mL). This was done because of the available supply of basal media. Serum composition was reduced to conserve this valuable material, and cells showed no decline in growth rate with less serum.

- CCD-1068SK fibroblast cells were cultured in a 75 cm^2 flask until ~90-100% confluence was reached, while HEP G2 and Vero cells were cultured to ~70-90% confluence. Fibroblasts were cultured in flasks to near complete confluence because of much lower cell count per flask. For example, a typical 75 cm^2 flask of MCF-7 yielded ~10-15 million cells, whereas a flask of the same size and level of confluence yielded 1-2 million CCD-1068SK cells. All cell types were tested in both hot and cold hydrogels dissolved in growth media.

Harvesting Cell Spheroids Method

- Allow culture plate to equilibrate to room temperature for gel to transition back to liquid.
- To expedite harvest, chill culture vessel on ice packs for 30 minutes to re-liquefy hydrogel.
- Pipette liquefied hydrogel containing cell spheroids to desired container.
- Alternatively, the liquefied hydrogel can be gently centrifuged to concentrate spheroids and remove majority of the hydrogel.
- Spheroids can now be seeded in a new culture vessel or used for experiment of choice (example: staining procedure).
- Hydrogels seeded in complete media revert back to two dimensional growth.

Immunofluorescent Staining of MCF-7 Spheroid for E-Cadherin Expression Method

Materials

DPBS- Dulbecco's PBS Gibco Ref# 14040-133 Lot: 1606175

BSA – Bovine Serum Albumin Sigma Aldrich A7906-50G Lot: SLBD8601V

Triton X-100 Sigma T8787-50 mL Lot MKBW1852V

Tween 20 Spectrum PO132 Lot: 1AH1006

Formaldehyde Sigma 47608-250 mL-F Lot: BCNQ3593V

10X PBS Sigma P5493-4 L batch: SLBH5342

BD Biocoat 8 Well Culture Slide Poly-D-Lysine/Laminin product 354688 Lot: 35219

Mouse anti-E-Cadherin (HECD-1) 2^{nd} GEN Predilute Ref 0812222 Lot: 1732006A 3.95 µg/mL

Alexa Fluor 488 goat anti-mouse IgG (H+L) Microprobes Ref: A11001 Lot: 172653 2 mg/mL

Staining buffer = 0.2% Triton X-100, 0.1% BSA, 0.05% Tween 20 in PBS

Blocking buffer = 3% BSA in staining buffer

Fixing Solution = 4% Formaldehyde, 0.1% Triton X-100 in PBS

- Harvested spheroids from thermogel by allowing well plate to equilibrate to room temperature and added cold Dulbecco's PBS (~50% of the volume of thermogel; i.e. 0.5 mL of PBS to 1.0 mL of thermogel)
- Pipetted liquefied thermogel mixture to 15 mL centrifuge tube and centrifuged briefly (~1 minute) at 1000 RPM.
- Removed as much liquid as possible without disturbing cells in bottom of tube.
- Added 0.5 mL of fixing solution to tube and allowed solution to sit on cells for ~30 minutes at room temperature.
- Centrifuged tube at 1000 RPM for 2 minutes then removed as much liquid as possible.
- Rinsed cells with 2.5 mL PBS and centrifuged briefly at 1000 RPM.

- Removed a portion of the PBS, ~1 mL, then suspended the cells in the remaining liquid and transferred 200 µL of this to BD Biocoat 8 well culture slide.
- Allowed cells in PBS to incubate at room temperature on a culture slide for ~30 minutes then removed liquid and added 200 µL of blocking buffer to wells of culture slide.
- Incubated for ~2 hours with blocking buffer, then removed blocking buffer and washed wells with 150 µL of PBS three times.
- Added 1 drop of primary antibody (~50 µL) as well as 100 µL of staining buffer and allowed to incubate for ~1 hour at room temperature.
- Removed primary antibody and washed wells with 150 µL of PBS three times.
- Added 200 µL of secondary antibody diluted to 2 µg/mL in staining buffer and allowed to incubate at room temperature for ~1 hour.
- Removed secondary antibody and washed wells with 150 µL of PBS three times.
- Then added one drop of Gel Mount aqueous mounting media with anti-fade.
- Waited ~10 minutes then imaged with blue filter on AMSCOPE IN300-FL

Note: Culture slide protected from light as much as possible during procedure from step 10 until microscope imaging complete.