Three-Dimensional Cell Culture Within A Biomimetic Hydrogel Based On Chitosan And Hyaluronan

John H. Brekke,* Jon Holy,** Matthew T. Stuart,*** Gregory E. Rutkowski *** Wilmar Salo,** Lee Sandquist ,**** Timothy D. O'Brien****

*Bioactive Regenerative Therapeutics, Inc (BRTI); **University of Minnesota Duluth – School of Medicine; ***University of Minnesota Duluth – Department of Chemical Engineering; ****University of Minnesota Stem Cell Institute, Mpls, MN

Introduction A growing demand for study of cells within biologically realistic, *in vitro,* microenvironments requires a paradigm shift in cell culture technology: from cell culture on 2-D, rigid substrata to cell growth within a 3-D space, defined by malleable materials composed of biologically "smart" compounds.[1, 2]

Therapeutic issues addressed by tissue engineering and regenerative medicine require similar materials, customized for human application in a wide variety of treatment circumstances: ranging from the ideal operating room to battle field conditions.

Any material answering these demands must possess the following properties:

1) Ability to maintain over time a defined, three-dimensional shape and size when saturated with cell suspension solution / media;

2) Adequate mass transfer capacity to serve cells throughout its internal spaces;

3) Ease of use: (a) rapid and atraumatic encapsulation of significant cell populations; (b) *in vivo* application via minimally invasive methods;

4) Be free of potentially hazardous cross linking or conjugating agents;

5) Be composed of elements capable of supporting the phenotypic end point for encapsulated cells;

6) Be composed of elements suitable for approval by the Food and Drug Administration for human application;

7) Be cost effective for the researcher and the clinician.

Materials Hyaluronan (HY^+) (Mw=400-600kDa; PD < 4.0) and chitosan (CT) (Mw = 400-600kDa; PD < 3.0) were chosen because of their established biologic properties favoring angiogenesis and support of the chondrocyte phenotype as well as their structural, mechanical and hydrologic properties.[3, 4] Hyaluronan is used in its acid form (HY^+) while CT is used in its base form, 85% deacetylated, and protonated with formic acid to 70% of available amines. Chitosan was received from NovaMatrix (Oslo, Norway) as filter sterilized, aseptically filled, 0.1% solution. HY^+ was received from Lifecore Biomedical (Chaska, MN, USA) as a filter sterilized, aseptically filled, 0.3% solution. The entire fabrication process is designed to maintain reactant sterility; no terminal sterilization is employed.

HY^+ and CT are separately lyophilized, reduced to small particles and blended together in dry form at a mass ratio of HY^+ = 1.0 : CT = 1.44. The acronym for this dry blend is HCP-h representing **H**yaluronan-**C**hitosan-**P**olyelectrolytic complex – **h**ydrogel. (Figure 1, stereomicroscopy insert.)

When used as a cell culture microenvironment, 30.0 mg (+/- 0.4 mg) of HCP-h is hydrated with the cell suspension solution at a ratio of 15 µL soln. / 1.0 mg HCP-h. The cell suspension solution consists of at least 420µL dextran (Mw = 40,000) in 5% dextrose and water (Abbott Laboratories) and a volume of cell population to equal 500µL total hydration solution.

When used as a platform for high concentration regional delivery of growth factors, morphogens, cytotoxic agents or other therapeutic compounds, sterile water solutions are used for HCP-h hydration.[5]

Once hydrated, HY^+ and CT particles of the HCP-h dry blend begin to dissolve and, where physically proximate to one another, form insoluble, polyelectrolytic complex (PEC) fibers at the nanometer scale.[6] This process begins at multiple locations, simultaneously, throughout the hydrated mass and concludes with these PEC fibers binding to each other, forming a microscopic, filamentous network around and through regions of unreacted HY^+ and CT. Cells suspended in this system become bound by the growing PEC fibers and their random interconnections.

Methods Figure 1 demonstrates the process for hydrating 30mg HCP-h with 500uL of dextran-cell suspension solution. Figure 2: Once the entire HPC-h mass has accepted its fluid of hydration, it is placed in the formation funnel and centrifuged at 1500g-1800g for 30-45 seconds (including g-force ramp up and down) to form a 4.4 mm diameter, malleable cylinder.

Fig. 1: Hydration of 30 mg HCP-h dry blend with 500uL dextran-cell suspension solution.

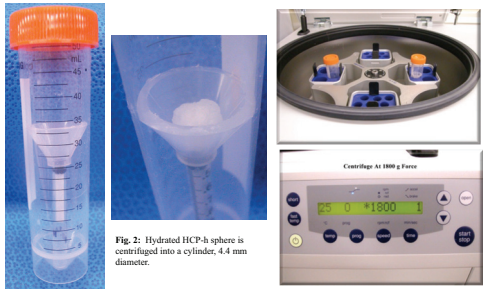


Fig. 2: Hydrated HCP-h sphere is centrifuged into a cylinder, 4.4 mm diameter.

The funnel is removed from the syringe barrel. The HCP-h material, now charged with a cell population up to 50 x 10^6 cell / ml, is expressed into media and cultured under agitation. Figure 3a and 3b.

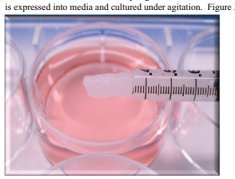


Fig. 3a

Fig. 3b

Insoluble HY^+ - CT polyelectrolytic complex fibers are shown at the nanometer scale in figure 4a (est. mag = 207kx) and as larger bands or cords of PEC fibers in figure 4b (SEM / mag. = 500x).

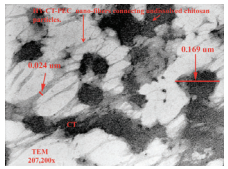


Fig. 4a: TEM-insoluble HY-CT-PEC fibers at the nanometer scale.

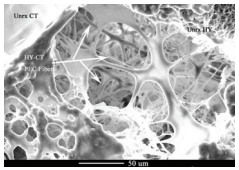


Fig. 4b: SEM-cords of PEC fibers. Original Mag: 500x

Results

Fluorescence microscopy

Cells embedded within the HCP-h "cocoon" may be studied by various light microscopy techniques including fluorescence microscopy and differential interference contrast (DIC) microscopy. Figure 5 demonstrates C-8161 malignant melanoma cells, encapsulated within a HCP-h "cocoon" at a population concentration of 50 x 10^6 cells / ml, following treatment with calcein-AM. The 8 µm thick specimen was taken from the center of a 4.4 mm HCP-h cylinder following flash freezing in liquid N_2 and sectioning on an ICE cryotome at -25•C. (Olympus BX-40 equipped with epifluorescence filters and Hg vapor light source.)

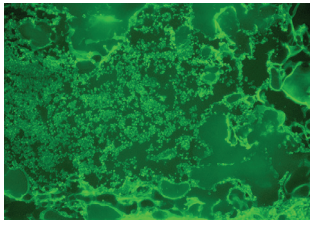


Fig. 5: Example of fluorescence microscopy applied to cells embedded within HCP-h "cocoon". Epifluorescence, calcein-AM, C-8161 malignant melanoma cells. Frozen section (8 µm section, center of "cocoon", 100x mag.)

Differential Interference Contrast (DIC) Microscopy

Figures 6a and 6b show C-8161 malignant melanoma cells cultured within an HCP-h cocoon 48 hrs. in DMEM / FCS. Fig 6a is a DIC phase contrast image showing (from left to right) a free standing HY-CT-PEC fiber, multiple cells clustered among additional HY-CT-PEC fibers, unreacted CT or HY material and finally another cluster of cells. Fig. 6b is a fluorescence microscopy image of the same field shown in fig. 6a showing the cells following treatment with Hoechst 33342 nuclear stain. HY-CT-PEC fibers appear as darkened spaces between the cells.

Fig. 6a: DIC Image, est. mag = 1,000x

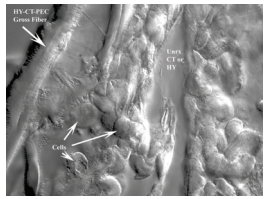
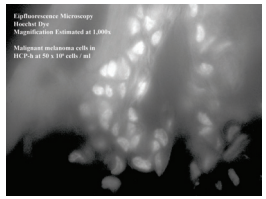


Fig. 6b: Hoechst 33342 Nuclear Stain, est. mag = 1,000x



HCP-h In situ RT-PCR

Mesenchymal stem cells derived from non-human primate bone marrow were expanded in 2-D culture and invested within a 1000uL HCP-h cocoon at a population concentration of 23 x 10^6 / ml. The cocoon was divided into 500uL segments and cultured in chondrogenic media containing transforming growth factor-beta-1 (TGF-B1), bone morphogenetic protein-4 (BMP-4) and insulin-like growth factor-1 (IGF1); all at 10ng / ml media. By culture day 7 both HCP-h cocoons produced evidence of messenger ribonucleic acid (mRNA) for Sox-9, aggrecan, collagen Type-IIa, and link protein (CRTL-1); all markers of the chondrocyte phenotype (figure 7a). Signals for these markers were detectable, but weak; due in large part to chitosan's (strong cation) ability to ionically bind negatively charged nucleic acids released from lysed cells.[7]

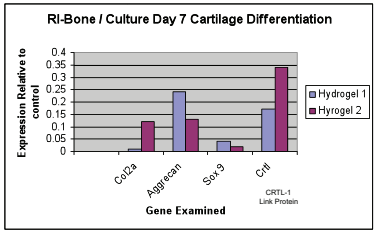


Fig. 7a: MSC induction to the chondrocyte phenotype within HCP-h "cocoons"

We have developed a nucleic acid extraction protocol employing cetyltrimethylammonium bromide (CETA) and polyvinylpyrolidone (PVP) to circumvent this problem.[8] CTAB neutralizes mRNA's negative charge while PVP masks chitosan's strong positive charge, thus allowing precipitation of mRNA in significant quantities.[8]

Figure 7b depicts an absorbance curve at 260nm showing 50µg yield of RNA extracted by CTAB / PVP buffer from 100µL HCP-h "cocoon" segment containing C-8161 malignant melanoma cells at a population concentration equivalent to 20 x 10^6 cells / ml.

Figure 7c is an electrophoresis gel demonstrating glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) gene segments derived from mRNA of glioblastoma cells. Lanes 3-6 were run with mRNA extracted using a standard (Trizol) extraction buffer; lanes 7-10 were run with mRNA obtained by the CTAB / PVP extraction buffer. mRNA retrieved from cells in monolayer culture (lanes 3,4 & 7,8) was compared with mRNA retrieved from cells cultured within HCP-h "cocoons" (lanes 5,6 & 9,10). CTAB / PVP buffer provided significant quantities of good quality, RT-PCR ready, mRNA from cells in both 2-D and 3-D culture systems.

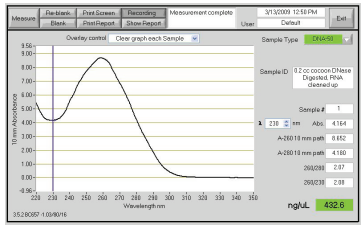


Fig. 7b: Nano Drop spectrophotometric curve of RNA sample extracted from C-8161 cells embedded within HCP-h cocoon. 260nm / 280 nm ratio = 2.02. **Gross yield = 21.6 µg RNA.**

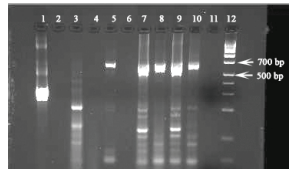


Fig. 7c: Electrophoresis gel of PCR product for GA3PDH control gene. Lanes 3-5 mRNA extracted by Trizol. Lanes 6-10 mRNA obtained by CTAB / PVP extraction buffer.

Conclusions A practical 3-D cell culture construct could serve as an *in vitro* surrogate for expensive and time consuming animal studies now required for development of new pharmaceuticals. Similarly, it may function as a platform for high concentration, regional delivery of drugs, growth factors, morphogens and other therapeutic agents. The proposed 3-D cell culture construct may also be the basis for determining rational adjustment of dosing regimens for different anti-cancer drug therapies. Finally, a 3-D cell culture material could function as an *in vitro* environment for differentiation of therapeutic phenotypes as well as a delivery vehicle for differentiated cells in human regenerative medicine applications.[1, 2]

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