Proceedings of the 28th IEEE
EMBS Annual International Conference
New York City, USA, Aug 30-Sept 3, 2006

A Novel Use for Capillary Channel Fibers: Enhanced Engineered Tissue Systems

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Abstract—**Capillary channel fibers (CCF) were selected for use in tissue engineering systems, due to their fluid wicking and cell guiding abilities. A study was conducted, comparing cell adhesion to CCF and mesh to traditional systems with fibers of round cross sectional area. A series of biochemical assays was conducted; the results indicated enhanced cellular attachment to the CCF based systems. Ongoing studies are being conducted to determine the rate of liquid diffusion within CCF reinforced tissue engineered composite systems.**

I. Introduction

Research direction initiated in our laboratory, based on our 2001 written disclosure, has led to the development of tissue engineered materials incorporating fibers with non-circular cross-section [1]. The concept stemmed from our assessment of the Eastman Kodak "4DG™" fiber intellectual property that was donated to Clemson University. The 4DG™ fibers are channeled substrates that allow wicking of fluid along their length through capillary action; traditional applications for such fibers include athletic wear, filters, and numerous other absorbent products where fluid movement is key. We quickly noted that these unique attributes would likely be conducive to the production of tissue engineered substrates, where wicking of liquids (nutrients and waste products) is crucial; i.e., the addition of these wicking fibers (known as "capillary channel fibers", or CCF) to tissue engineered substrates would cause enhanced diffusion. Additionally we hypothesized that the fiber morphology would allow the improved attachment and alignment of cells. Like other devices, the fibers (and/or fiber channels) could be loaded with therapeutics for timed release. The fibers may indeed be useful in the establishment of vascular networks and the long term viability of "thick" tissue constructs.

With the goal of providing a viable tissue construct, an injectable composite system was developed in our laboratory whereby cells are seeded on filler materials (beads or fibers) and subsequently suspended within a hydrogel carrier medium, resulting in a cellular scaffold that may be injected into a patient through a syringe [2, 3]. Anchorage dependent cells are generally function limited when embedded directly in a gel, thus a composite system affords an environment that is conducive to cellular proliferation, but which needs refinement in order to optimize fluid transport to and from the incorporated cells.

Manuscript received May 24, 2006. This work was supported in part by the U.S. National Science Foundation under a Presidential Early Career Award for Scientists and Engineers and a Department of Defense Era of Hope Scholarship.

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II. Methods

A preliminary four day study was conducted to determine the effect of CCF based poly-L-lactide (PLLA) mesh on cellular attachment. Four woven mesh scaffolds were tested, each having a scaffold size of 38.1 mm by 38.1 mm.

The first step of the study was to prepare the monofilaments to be used in the production of mesh. PLLA pellets (Boehringer Ingelheim) were weighed out in 4-5 mg allotments. Monofilaments were extruded from the pellets using a ThermoHaake Minilab Rheomex CTW5 extruder (Thermo Electron Corporation).

Four dies were designed and fabricated by Clemson University Machining and Technical Services for use in the extruder to produce CCF and control fibers. The dies were applied to produce CCF and circular fibers of 0.5mm diameter and 1.25mm. The extrusion speed was 60 revolutions per minute (RPM) at 210°C and the drawing unit, with a diameter of 127 mm, was set to either 6 or 12 RPM. The 4-5 mg weighed samples were loaded into the hopper, packed into the twin-screw extrusion chamber, and mixed for 3 minutes at 210°C. The PLLA pellets were extruded through the dies to form the monofilaments. The monofilaments were stored in a desiccator under vacuum and devoid of light until use.

The monofilaments were evaluated prior to cell culture to determine polymer and monofilament consistency using standard histological procedures, differential scanning calorimetry (DSC), and gel permeation chromatography (GPC). The extruded fibers were excised into 2 cm sections from the beginning, middle and end of the monofilament with sterile stainless steel #11 surgical blades for use with a no. 3 handle (Feather) and were embedded in a Technovit 7100 glycol methacrylate (GMA) embedding solution obtained from Kulzer. An amount of 100 ml of base

solution was combined with 1 g of Hardner I for 5 minutes. The embedding solution was formed by stirring 15 ml of the preparation solution with 1 ml of Hardener II for one minute.

The 2 cm sections of monofilament were embedded within the GMA embedding solution in peel-away molds and block holders with hollow centers. The fibers were placed down through the peel-away molds and block holders to align the fibers in an upright position. The well was then filled with embedding solution. The GMA embedding solution was allowed to polymerize at room temperature for one hour. The samples were then transferred to a 37°C walk-in heated room for one hour to intensify the polymerization. The samples were stored overnight in an empty desiccator to minimize water concentration in the hydrophilic GMA embedding solution.

The samples were sectioned at 5 μm on a Leica RM2155 automated rotary microtome. The sections were placed into a 25°C distilled water bath (Baxter Scientific Products) containing no residual soap or oil. The water bath caused the hydrophilic GMA to spread out into wrinkle-free sections. A clean slide (VWR International) was used to retrieve the sections. The slide was allowed to dry for a minimum of 2 hours at 37°C. The slides were cover slipped with xylene-based Cytoseal XYL (Richard-Allen Scientific). An inverted Axiovert 135 microscope (Zeiss) and an attached digital Spot camera (Diagnostic Instruments) were used to acquire and analyze images. The perimeter and area of the sections were determined.

Differential Scanning Calorimetry (DSC) (DSC 7 with PYRIS™ software: Perkin Elmer) was performed in triplicate on the as-received material and the fibers at several points, in an inert nitrogenous condition and using indium calibration standards. Specifically, the thermal properties such as the glass transition (T_g), melting temperature (T_m) and crystallinity temperature (T_c), as well as the corresponding enthalpy changes of the PLLA monofilament were monitored.

This materials analysis allowed the tracking of degradation of the polymer through the processing, sterilization, and incubation period. Each sample (5-10mg) was heated from 25ºC to 220ºC at a ramp rate of 10ºC/min, quenched to 25ºC, following which the heating cycle was repeated from 25ºC to 220ºC at a ramp rate of 10ºC/min. GPC was conducted using a Waters HPLC system consisting of a Model 610 HPLC pump, a Waters Model 486 UV/Vis detector, and a Waters model U6K injector. Samples and standards were prepared in HPLC grade chloroform (Honeywell Burdick and Jackson) at a concentration of 3mg/mL. Polystyrene standards of molecular weights of 1000000, 400000, 230000, 104000, 50000, 23000, 4000, with M_W/M_N, polydispersity, values of 1.1 or less (PolySciences, Inc., Warrington, PA) and a 436 standard which has M_W/M_N of 1.16 (Polymer Standards Service – USA, Inc.) were used. Samples and standards were filtered through ACRODISC® CR 13mm Syringe Filters with 0.2μm PTFE membrane (PALL Gelman Laboratory). Sample volumes of 50μL were introduced into the U6K injector using a Hamilton 50μL syringe (Hamilton Company) and samples and standards were eluted at a flow rate of 1.0mL/minute through two PLgel, 5μm, Mixed bed-C columns (Polymer Laboratories, Ltd.). Samples and standards absorbance was detected at 254 nm and data was analyzed using Waters Millennium 32 chromatography software based on elution times and the standard curve.

Scaffolds were fabricated using the extruded PLLA monofilaments. Specifically, four mesh scaffolds, two comprised of large CCF and two comprised of large round fibers, were prepared according to the following procedure. The extruded PLLA monofilaments were cut to the desired length with sterile, stainless steel #11 surgical blades for use with a no. 3 handle. Additionally, 270 sections of both the round and lobed monofilaments were cut to lengths of 2 cm for use in a four week stir flask cell attachment study. Other sections were cut to a length of 76.2 mm. These longer sections were hand-woven into a mesh using a cork board and push pins to maintain a double fiber square weave pattern. In order to maintain the mesh structure during the cell culture period, the material edges were soldered with a spatula heated by a Bunsen burner to form 76.2 mm x 76.2 mm square mesh structures. All scaffolds, fibers, and mesh were stored in a desiccator under vacuum and devoid of light until use in culture.

The mesh scaffolds were placed in individual 88.9 mm x 203.2 mm Converters Self-Seal Pouches (Cardinal Health) for sterilization with ethylene oxide (AN74i ANPROLENE®; Anderson Products) and degassed under vacuum for 48 hours. Following the degassing, the pouches were stored in an empty desiccator until use.

After scaffold development and sterilization, 3T3M fibroblasts were cultured in Dulbecco's Modified Eagle Medium (Invitrogen Corporation) supplemented with 50mL Fetal Bovine Serum (FBS), USDA-Cert (Mediatech, Inc.), 5mL L-glutamine – 200mM (Invitrogen Corp.), 1mL Fungizone Amphotericin B (Invitrogen Corp.), 5mL Antimycotic/Antibiotic (Invitrogen Corp.), and 5μg bovine Fibroblast Growth Factor (EMD Biosciences, Inc.). Cultures were maintained at 37°C and 5% CO_2 (Sanyo Electric Biomedical Co., Ltd., Model: MCO-17AIC).

Stir flasks of volume 250 mL (CELSTIR® Flask, Wheaton, Millville, NJ) were coated with 8 mL of non-

sterile SIGMACOTE® (Sigma-Aldrich, St. Louis, MO). Excess SIGMACOTE® was discarded, and the flasks were allowed to air dry for two hours before autoclaving. A total of 180 fibers were used for the cellular attachment study: 90 (45 round and 45 CCF) were seeded with 5.0E5 3T3M fibroblasts per 250mL Celstir® and the remaining 90 (45 round and 45 CCF) acellular served as controls. The flasks were each filled with 50mL of media to soak the fibers for 24 hours and placed on a stir plate at 45 rpm (Corning Stirrer/Hot Plate, model PC-420 and Thermolyne 45700 CELLGRO™ Stirrer, model S45725) in the incubator at 37°C and 5% CO_2 (Sanyo Electric Biomedical Co., Ltd., Model: MCO-17AIC). After the 24 hour incubation period, three flasks of each fiber type were seeded with 5.0E5 3T3M fibroblasts and left to incubate at the same conditions for an additional 24 hours. After the additional 24 hours, 100mL of medium was added to all flasks to bring the total volume in each flask to 150mL.

Next, four cell-seeded PLLA sterile mesh (two large CCF and two large round fiber) were obtained by using presterilized flatbed biochambers with 0.1 μm pore PET membrane and presterilized disposable flow path, DIFS (Tissue Genesis, Inc.). Mesh were placed in MEDIA-MISER® dishes (Fisher Scientific) and covered with 8 mL of 3T3-M supplemented medium. The mesh was allowed to soak for 1.5 hours in a CO_2 incubator (Sanyo Electrical Biomedical Co., Ltd., Model: MCO-17AIC) at 5% CO_2 and 37ºC. Sterile retractors were used to separate the top cover of the biochamber, and the mesh was transferred with sterile forceps from MEDIA-MISER® dishes into the bottom compartment, the tissue culture treated poly-styrene base, of the biochamber.

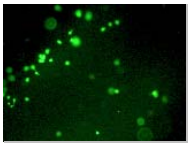
One T-150 flask (CORNING®) of 3T3M fibroblasts, passage 29, was trypsinized, (Invitrogen Corporation), and each scaffold was seeded with 1 mL of medium containing 3.96E6 cells. After seeding, the biochambers were re-sealed, fastened within the biochamber clamp and placed in the incubator. The monofilaments were evaluated for cellular response at 2, 14 and 28 days and the mesh scaffolds were evaluated every day for a four day study. Cellular metabolism, viability/cytotoxicity, and histological techniques were used to determine cellular activity. Every day the cartridges were removed from the incubator for examination and pictures of the cellular attachment progress on the mesh scaffolds were made with an inverted microscope.

Glucose consumption and lactic acid production by the cells indicated the metabolic activity of the system. Glucose consumption and lactic acid production was measured in the cell medium according to the following procedure. Medium aliquots of 0.2 mL were taken in triplicate every 24 hours. Lactic acid and glucose measurements were made using a biochemistry analyzer (YSI).

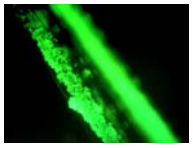
LIVE/DEAD® Viability/Cytotoxicity Kit (L-3234; Molecular Probes), an end point assay, was used to identify viable cells on the control chamber and experimental mesh after four days. Separate fibers were used at each time point. Three fibers were transferred to a sterile six-well plate, rinsed with 1 mL of Dulbecco's Phosphate Buffered Saline (PBS) (Sigma, St. Louis, MO). The PBS was aspirated, and 1.0 mL LIVE/DEAD® reagent was added to each well. The samples were protected from the light for 45 minutes at room temperature. Images were acquired with an inverted microscope (Zeiss, model: Axiovert 135) and attached digital Spot camera (Diagnostic Instruments). Images were taken with a 10x objective with an ATTOARC®2 HBO 100W light source (Zeiss).

III. Results

Results show that the glucose levels in the stir flasks with lobed fibers were significantly lower on days 8-21 and on day 28. The results also showed that the lactic acid levels in the stir flasks with lobed fibers were significantly higher on days 10-21, on day 24, and on day 28. Metabolic activity was linearly related to the number of 3T3M fibroblasts in monolayer culture. Metabolic testing indicated that the mean metabolic activity of the fibroblasts cultured on CCF was greater than the metabolic activity of the fibroblasts cultured on round fibers at two days, two weeks, and four weeks. There was no statistically significant difference between any group after two days, but the metabolic activity of the cells housed on the CCF was statistically higher than the round monofilament, after both two weeks and four weeks. The mean metabolic activity of cells housed on the round fibers was positive, but it was not significant as compared to the lobed control or round control at any point in the study. There was a significant increase in the metabolic activity of the cells on the lobed monofilaments from two days to four weeks, but there was not a significant increase for cells on the round monofilaments between time points.



Round fiber with low density of cells



CCF with high density of cells

Material properties of the PLLA were measured. The as-received PLLA pellets were in crystalline form. The inherent viscosity of the as-received pellets was measured to be 5.43±0.02 dL/g. This inherent viscosity significantly lowered during the first ten days of cell culture. The number average molecular weight (M_N), weight average molecular weight (M_W), and the molecular weight peak (M_P), of the PLLA pellets exceeded the bounds of the GPC. The number

average, weight average, and the molecular peak of the PLLA were quantifiable post processing, and the round fiber measurements were significantly larger than the CCF. The polydispersity measurements between the fiber types were not significantly different post processing. The crystallization temperature (T_c) was not observed in the as-received pellet, yet was observed post processing for both monofilaments. Post-processing, the T_m was observed to be more bimodal for both monofilaments.

IV. Conclusions

The research described here sets the foundation for the use of 4DG or equivalent wicking fibers in multiple tissue engineered systems, including bone, tendon, cartilage, breast, fat tissue repairs. We are currently conducting studies to assess the differences in diffusion in composite systems with and without CCF filler. The CCF, or wicking fibers, may indeed provide the development of "thick" tissue constructs that are clinically relevant.

Acknowledgment

K. J. L. Burg thanks Tissue Genesis, Inc. for the use of their bioreactor systems and Dr. Mark Saltzman of Yale University for his assistance with diffusion studies.

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