Fabrication of a multi-layer three-dimensional scaffold with controlled porous micro-architecture for application in small intestine tissue engineering

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Various methods can be employed to fabricate scaffolds with characteristics that promote cell-to-material interaction. This report examines the use of a novel technique combining compression molding with particulate leaching to create a unique multi-layered scaffold with differential porosities and pore sizes that provides a high level of control to influence cell behavior. These cell behavioral responses were primarily characterized by bridging and penetration of two cell types (epithelial and smooth muscle cells) on the scaffold in vitro. Larger pore sizes corresponded to an increase in pore penetration, and a decrease in pore bridging. In addition, smaller cells (epithelial) penetrated further into the scaffold than larger cells (smooth muscle cells). In vivo evaluation of a multi-layered scaffold was well tolerated for 75 d in a rodent model. This data shows the ability of the components of multi-layered scaffolds to influence cell behavior, and demonstrates the potential for these scaffolds to promote desired tissue outcomes in vivo.

Introduction

Tissue engineering principles have successfully been used to develop implantable cell/biomaterial constructs for reconstructing the bladder, a hollow organ with laminar wall architecture.1-4 In these examples, de novo organogenesis was catalyzed following implantation of the constructs and resulted in the regeneration of a functional organ.1-4 Various attempts have also been made to engineer the small intestine (SI), another example of a tubular organ with laminar wall architecture.5 We have successfully applied methodologies developed to mediate bladder regeneration toward regeneration of SI tissue.6 Such approaches suggest a potential treatment for short bowel syndrome (SBS). SBS stems from surgical removal or disease of the SI, leading to malabsorption of nutrients.7,8 Improving the patient’s nutrition through enteral and parenteral feeding has been a helpful treatment for patients with SBS; however, there is high morbidity and high cost associated with parenteral treatment.9 Current surgical procedures used to treat SBS include increasing the length of the intestinal loop as well as intestinal transplantation. Procedures to increase the length of intestinal loop have not met with much success with long-term survival rates at 45% in pediatric patients9 and complications such as fistula formation and sepsis in adults.10 With intestinal transplantation, issues include graft rejection, limited availability of organ donors, and poor patient survival rates,7 indicating a need for better treatment options of SBS.

For tissue engineering of the SI, a three-dimensional (3D) scaffold is required to deliver/recruit appropriate cells as well as provide a 3D template for organization of the regenerating tissue. The role of the scaffold is critical to support the biological and mechanical needs of the regenerating tissue. The interaction of cells with a scaffold surface can affect cell behavior. This behavior includes cell attachment, migration, proliferation, and differentiation to a desired cell phenotype, all which can influence the rate and quality of tissue formation.11,12 It is therefore critical to design the scaffold to provide a conducive environment for cell/material interactions. Surface topography is a key element in achieving such an environment. Such topographies can be created by a variety of methods including electrospinning, phase separation, and precipitation as well as gas foaming/particulate leaching.12-15

In this report, we describe a novel methodology that combines compression molding with solvent casting/particulate leaching to develop a novel, multi-layered scaffold. The resulting tubular structure has different porosities and pore sizes in each layer to achieve such an environment. Such topographies can be created by a variety of methods including electrospinning, phase separation, and precipitation as well as gas foaming/particulate leaching.12-15

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molecules may be possible. In our studies, we observed that certain scaffold characteristics such as pore size can influence pore bridging and pore penetration by cells in vitro. Bridging refers to the distance cells on the surface of the scaffold extend over a pore to cover pore openings, while penetration refers to the distance a cell migrates into the scaffold from its surface. Both bridging and penetration can promote the formation of different cell layers within the construct composite (illustrated in Fig. 1).

The generation of multiple cell layers in a scaffold can be useful in tissue engineering as the result would more closely approximate native tissues that possess layered structures that fulfill various biological and mechanical needs. Finally, we assessed host bioresponse to multi-layer tubular scaffolds up to 75 d post-implantation. We observed no evidence of residual scaffold and noted a replacement wall comprised of fibrovascular rich matrix, minimal inflammation, partial re-epithelialization and focal infiltration of smooth muscle bundles. Taken together, these data indicate that scaffold porosity and pore size can be manipulated within a multi-layer scaffold and that these types of structures influence cell behavior. When seeded with cells and implanted, these well-tolerated 3D scaffolds have the potential to lead to regenerative outcomes that more closely resemble native tissue structures.

**Results**

**Appearance of multi-layered polymer tubes.** The morphology of multi-layered tubes generated using a solvent-cast particulate-leached compression molding technique was determined by SEM. Figure 2 shows a representative image detailing the morphology of a mesh-reinforced two-layer polymer tube. Both the inner and outer layers and the intervening knitted mesh are visible. Also, strikingly, is the progression of different pore structures (porosities/pore sizes) incorporated into the tube fabrication, as well as good pore interconnectivity between layers as previously described. Overall, the tube was intact and the layers were seamlessly joined together.

**Influence of pore structure on cell bridging and penetration.** Both IEC and Ad-SMC adapted well in culture to the scaffold surfaces and bridged across pores in a number of constructs, as shown in a representative SEM image (Fig. 3).

The effect of the pores on cell bridging was analyzed using linear modeling. A full factorial model was developed using pore size and porosity to significantly predict SEM bridging for both cell types. Figure 4A and B shows the surface profile graphs for IEC and Ad-SMC and the impact of cell type, porosity (z-axis) and pore size (x-axis) on cell bridging as measured by SEM (y-axis). The results suggest a clear tendency for increased cell bridging, of either cell type, with decreasing pore size. In contrast, varying porosity did not have a measurable impact on cell bridging.

To quantify cell penetration within pores, confocal imaging was performed. A direct relationship between penetration and pore size was hypothesized by which an increased depth was gained with an increase in pore dimensions. We confirmed this expectation, as shown in Figure 5 for both cell types, with an increase in penetration with increasing pore size. Furthermore, the cell type plays an important role in this penetration characteristic, as the IEC traveled significantly deeper within the pore structures than did the Ad-SMC as shown in Figure 5 ($P < 0.05$, $n = 5$ for each cell type).

**Histological observations.** An acellular multi-layered polymer scaffold tube was evaluated histologically 75 d post-implantation. Evaluation of the implant at the defect site (longitudinal
and transverse sections) showed no evidence of residual biomaterial. Replacement tissue (intestinal wall) at the defect site showed nearly complete re-epithelialization of the luminal mucosal surface as shown in Figure 6. Furthermore, it also shows that tissue was comprised of a fibro-vascular rich matrix with minimal detectable remnant chronic inflammation. However, little infiltration of smooth muscle cells was detected at the margins of the anastomosis site. Necrosis was minimally observed as characterized by focal epithelial erosion. Acute inflammation was noted along with bacterial colonization, accounting for the lack of full re-epithelialization.

**Discussion**

This study reports on the development of a novel multi-layered tubular polymer scaffold, with the potential for providing a range of well-defined pore sizes and porosities within each layer that is achieved through a unique combination of solvent-casting/particulate-leaching with compression-molding. As observed by SEM (Fig. 2), there was a high degree of inter-connectivity between pores and smooth transitions between layers of different porosities, properties that can be easily controlled by the size and quantity of the porogen (e.g., salt crystal in this study; ref. 20). We show the influence of pore size on bridging and penetration by two representative cell types found in tubular or hollow tissues, epithelial, and smooth muscle cells (Figs. 3, 4, and 5). This supports earlier findings by Pamula et al. where they saw scaffolds with the largest pore diameter were best suited for the colonization of osteogenic cells.15,21 Furthermore, an increase in scaffold pore size was associated with an increase in depth of cellular penetration (Fig. 5). We hypothesize that the deeper penetration into the scaffold with increased pore size could result from the macroscopic inter-connectivity between pores, which would allow for greater cell penetration through the scaffold.22 Within a scaffold, a suitable pore size should allow enough surface area for cells to attach and proliferate, resulting in sufficient permeability23 to allow for the transport of nutrients, signaling molecules and waste products. Conversely, the lack of a suitable pore size could prevent vascularization of the scaffold in vivo, with the potential for inflammation and fibrosis in the developing tissue.24 Additionally, a smaller pore size could limit cell infiltration, resulting in cellular capsule formation around the edges of the scaffold, which could hinder diffusion leading to necrosis within the scaffold.25

With the role of scaffolds in tissue engineering to provide a template for the organization of regenerating tissue, the use of multi-layered implants would likely target different cell population types to different layers of the composite scaffold, as predicted from this study for a SI application. We saw that the distance traveled by the IEC through the IEC scaffolds was greater than that of the Ad-SMC, which may have the tendency to bridge the pores due to their cell length.

In addition to cell speed and size, cell migration can be affected by many factors such as temperature, chemoattractants, and the mechanical properties of the cell substrates. The variation in material thickness that results from pore structure may impact mechanical properties of the substrate.34 The mechanism by which these factors influence cell migration involves modulation of signal transduction pathways leading to rearrangement of the cell cytoskeleton.31 Taken together, these data demonstrate the inter-relatedness of interconnectivity, speed, scaffold mechanical properties and pore structures.

The findings reported here provide information on scaffold design that would allow cells (such as epithelial cells) to bridge pores, thus remaining on the scaffold surface, (e.g., with pores smaller than 23 μm). While permitting other cells (such as smooth muscle cells) to exhibit multiple behaviors, such as to penetrate, bridge and form various tissue layers through the thickness of the scaffold. In addition to cell sizes, different cell

![Figure 2. Morphology of a two-layered polymer foam scaffold tube as shown by SEM. (A) Outer layer (80% porosity, 150–250 μm pore size); reinforcing mesh (shown by asterisks); inner layer (70% porosity, 23–53 μm pore size). 30× magnification, scale bar, 1 mm. (B) Higher magnification image (106×) of corresponding boxed area, scale bar, 0.1 mm.](image-url)
Host response to the biomaterial was assessed by implantation of an acellular multi-layered scaffold into the SI in a rat. Our laboratory has reported that acellular scaffolds, used in applications to augment or replace hollow or tubular urologic organs, are expected to primarily elicit reparative rather regenerative outcomes. The former is characterized by a fibrotic outcome with minimal vascularization vs. the latter with a more vascularized smooth muscle-like parenchyma. Consistent with these earlier observations, our studies showed a principally reparative response upon implantation of an acellular scaffold. On the other hand, additional work in our laboratory has shown that when patches and tubular scaffolds are seeded with Ad-SMC, and implanted within rodent models by direct anastomosis to host SI, they do facilitate the regeneration of SI tissue with complete regeneration of the epithelial layer and partial to full regeneration of the smooth muscle layer in 8–20 wks post-implantation. The use of Ad-SMC for our in vitro studies was based on this previous work that suggests Ad-SMC represents a source of smooth muscle cell population applicable in tissue engineering. The choice to use types can respond to differences in pore sizes as shown in the study by Mills et al., where they found that cells that migrate by cell-cell contact respond to the differences in pore sizes and cells that migrate as single cells do not.

In contrast to pore size, porosity showed no measurable influence on cell properties in vitro though it has the potential to influence the mechanical properties of the scaffold by affecting its strength. Having more pores in a scaffold results in more surface area available for cell growth and proliferation but limits the integrity and the ability for the scaffold to support load. The same reasoning could be applied to a scaffold with fewer pores, which would result in less surface area for cell growth but a much more stable scaffold. Guarino et al. noted this trend in their study, where they observed an increase in elastic modulus with a decrease in porosity. Porosity also has the ability to influence the degradation of a scaffold because a higher porosity means less scaffold material present, which could lead to a shorter degradation time. The techniques reported here have the potential to allow the design of a scaffold to control these properties.

Figure 3. Smooth muscle cells (Ad-SMC) on polymer scaffold disc, 163× magnification, scale bar 0.1 mm. Top panel, 60% porosity, 23–53 μm pore size scaffold disc; unseeded (A) or cell seeded (B). Bottom panel, 70% porosity, 53–150 μm pore size scaffold disc; unseeded (C) or cell seeded (D). White dashed circles represent single cells. IEC had similar responses (data not shown).
IEC was made because they are present in the SI organ and was used as a model to illustrate seeding with two different cell types with different characteristics. Similar to other tubular organs, two cell types may not be a requirement for the regeneration of such organs. Specifically, a scaffold could be implanted without IEC, which would then infiltrate from native tissue. Based on our studies, we speculate that cell-seeded scaffolds with the specialized, directive architecture presented here might facilitate smooth muscle tissue formation with the newly formed smooth muscle layer reflecting organization of native tissue by channeling host-derived or seeded cell populations through a pre-established foundation for cell migration and proliferation. Studies are currently underway to evaluate cell migration through these multi-layered scaffolds in vivo.

In conclusion, we have presented a technique to fabricate a 3D multi-layered scaffold with controlled and organized pore size and layered porosity. In addition, this study has demonstrated the ability of this scaffold type to influence cellular response through bridging and penetration. This novel methodology provides the ability to create scaffolds with specific architecture to promote regenerative responses in target tissue, and to potentially improve clinical outcomes.

Materials and Methods

Fabrication of polymer scaffold sheet and discs. Polycaprolactone (PCL, Sigma-Aldrich) $M_w < 160\,000$ Da and poly(lactic-co-glycolic acid) (PLGA, Lactel) $M_w \sim 32\,250$ Da were each dissolved in methylene chloride (Spectrum Chemical) to concentrations of 10% w/v. Salt crystals (Sigma-Aldrich) were milled and sieved to separate them by size. These salt crystals targeted the product of pore size diameters within the ranges of $23–53\,\mu$m, $53–150\,\mu$m, and $150–250\,\mu$m (sizes represent a physiological range within the body; ref. 37). The PCL/PLGA solutions and the salt crystals were combined in proportions that yielded a PCL to PLGA mass ratio of 9 to 1, and salt to polymer volume fractions (porosity) of 60%, 70%, and 80%. In a chemical fume hood, the resulting slurry was placed into a polytetrafluoroethylene (PTFE) mold, covered with a glass petri dish, and the solvent allowed to evaporate overnight. Polymer/salt composites were processed into sheets of $500–800\,\mu$m thickness using a roller press with parallel metal rollers that was heated to 70 °C (above the glass transition temperature of individual polymers). Sheets with five different pore structures (1 pore structure per sheet with combinations of porosity and pore size) were manufactured: 60%/23–53 μm, 60%/150–250 μm, 70%/53–150 μm, 80%/23–53 μm, and 80%/150–250 μm. From warmed (70 °C) polymer/salt composite sheets, discs were cut using a 5 mm circular punch.

Formation of multi-layered tubes. Polymer/salt composite sheets were layered using a series of custom-built molds to form tubes with final dimensions of 4 mm internal diameter and 1 mm wall thickness, which is based on the dimensions of the rat small intestine (unpublished data). Briefly, two or more sheets were heated to 70 °C and pressed together using a roller press until the total thickness was about 1 mm, resulting in the construction of multi-layered sheets. These sheets were wrapped around a 4 mm-diameter stainless steel mandrel, trimmed, and then placed into a 6 mm-diameter PTFE clamshell mold that kept the mandrel centered. A layered tube typically had dimensions of 4 mm-internal diameter, 6 mm-outter diameter, and up to 3 cm in length.

Alternatively, a reinforcing mesh was included in the construction of multi-layered scaffold tubes. The open-knit mesh (244–322 μm, Vicryl® VKML sheet, Ethicon) added stability and improved suture retention, particularly for tubes with higher porosity and larger pore sizes which tend to be more friable. Briefly, the reinforcing mesh was
wrapped around the inner tube, with the outer layer shaped by slicing a larger tube longitudinally and placing it around the inner layer and the mesh. The composite tube was placed into a heated aluminum mold and compressed longitudinally under high pressure, bonding the inner and outer layers through the open-knit mesh.

**Preparation of final polymer scaffolds by salt leaching.** The salt porogen was removed from the polymer/salt composites by submerging the material into distilled (DI) water at room temperature with gentle agitation for at least 36 h. The water was removed and replaced with fresh DI water once during the process. The resulting polymer scaffold pieces (discs or tubes) were rinsed with DI water, dried (in air then vacuum) and stored in a dry gas desiccator with 5% relative humidity at room temperature until use.

**Preparation of cell-seeded polymer scaffold constructs.** Polymer scaffolds containing one of the two cell types were prepared. Primary adipose-derived smooth muscle cells (Ad-SMC) were isolated and cultured in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum as previously described. Intestinal cells (IEC; cell line CRL-1592, ATCC) were cultured in medium supplemented with 0.1 U/ml of bovine insulin. Polymer discs containing any of the five pore structures were seeded at a density of 1.0 to 2.0 × 10^5 cells/5 mm disc (determined a priori to provide sufficient cell distribution across the scaffold surface at the five pore structures, and consistent with prior work) and cultured at 37 °C with 5% CO₂ over a period of 7 d prior to use.

**Morphology of multi-layered scaffold.** To observe their morphology, multi-layered scaffold tubes were placed on carbon-taped stubs, sputter-coated and imaged with a Philips 515 scanning electron microscope (SEM).

**Imaging of cell behavior on polymer scaffolds.** Images from SEM and confocal microscopy captured the two cell types’ behavior on single-layer polymer discs with each of five pore structures. The duplicate seeded discs (constructs) were fixed separately in 2.5% glutaraldehyde for SEM imaging with a Philips 515 unit or 2.5% formaldehyde for immunofluorescence imaging with a Zeiss LSM510 laser scanning confocal system.

For SEM, the samples were processed routinely at the Imaging core facility at Wake Forest Baptist Health Medical Center. The digital images of the surface and cross-sectional areas of the constructs were analyzed for bridging interactions of the two cell types with the pore structures. Multiple images were analyzed for every sample, with each representative of the surface area of the scaffold (~12% at 81.5× magnification, and 4% at 163×). We scored each image for the presence of cells bridged across pores using a semi-quantitative scale of 1 to 5, with '1' indicating partial coverage (<20%) of all pores in the field, and '5' associated with full coverage of all pores (>80%).

Confocal imaging of immunofluorescent-labeled cells (Live/Dead® Viability/Cytotoxicity kit, Life Technologies) was performed at the Imaging core facility (Wake Forest Baptist Health), and used to document cell penetration within pores. Each representative image was taken from a z-stack series tracking the full length and depth of cell penetration within the scaffold pores. The instrument software compiled penetration measurements for each cell type across the range of pore structures.

**Evaluation of bioresponse to multi-layered polymer scaffolds.** In vivo implantation of a tubular scaffold in a SI application was performed for evaluation of host bioresponse toward the scaffold. To this end, an acellular 1 cm long, three-layer tubular PCL scaffold (without reinforcing mesh) was constructed, with 60%/23–53 μm pore structure within the inner layer, to 70%/53–150 μm within the middle layer and 80%/150–250 μm within the outer layer was implanted in a Sprague Dawley male rat, 3–6 mo of age. Under anesthesia, the rat underwent a lower midline abdominal laparotomy and the SI was exposed. An approximately 1 cm-long section of the SI was removed and replaced with the implant, inserted between the intestine ends using an end-to-end anastomosis with continuous sutures of non-absorbable 7-0 silk to facilitate later detection of the implant location as described previously. The implant was covered with omentum to serve as a source of vascularization. The rat was initially restricted (7-d period) to soft foods and water intake, and thereafter allowed hard rat chow and water ad libitum. At 75 d post-implantation, the rat was euthanized and tissue was harvested for histological evaluation of the implant site. The study was conducted under approved Carolinas Medical Center Institutional Animal Care and Use Committee protocol number 10-09-03A.

**Histological evaluation.** At necropsy, the harvested tissue was placed in 10% buffered formalin for 48 h prior to obtaining longitudinal and transverse sections through the implant site. The sections were embedded in paraffin, from which 5 μm thick sections, mounted on charged slides were processed for hematoxylin and eosin (H&E) and Masson’s trichrome (Premier Laboratory LLC). The section slides were observed by light microscopy at three magnifications (100×, 200×, and 400×) to provide a semi-quantitative basic tissue responses and cellular integration. Tissue changes were assessed using severity grade scheme (grades 1, 2, 3, and 4) to which the descriptive terms (minimal, mild, moderate, and marked) were applied to determine the degree of
inflammation, vascularization, presence of muscular layer, luminal surface epithelialization, fibrosis, necrosis, calcification, and luminal patency as described. Assessed the presence of the biomaterial was performed at lowest magnification (10× and 40×), using polarized light by examining the birefringent property of the scaffold (PCL).

Statistical analysis. The factors of cell type, porosity, and pore size were used to predict the responses of cell bridging by SEM and penetration as observed by confocal microscopy. Numerical data were reported as the mean ± standard deviation where applicable. Post hoc statistical analyses of profiling (multi-parameter linear modeling) and variance (pair-wise comparisons) were performed with commercial software (JMP®, SAS). Differences were considered significant at $P < 0.05$.

Disclosure of Potential Conflicts of Interest
All authors declare an equity interest in Tengion, Inc.

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