Multi-Material Scaffolds for Tissue Engineering

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Summary: A trend in developing biocompatible scaffolds for tissue engineering has been to seek an ideal single material for which a given cell type will exhibit favorable behavior. While an ideal single material has proven elusive, scaffold manufacture using combinations of specialist materials can produce more versatile structures. By controlling the percentage and architecture of material components, mechanical properties, cell attachment, and proliferation may be optimized for a given function. Three specialist materials, poly-ε-caprolactone (PCL), fibrin, and alginate, were incorporated into multi-component scaffolds for a series of experiments testing each component with culture of fibroblasts. The rigid and formable PCL provided structure, the fibrin pore-filler allowed for cell attachment, and alginate thread provided a nutrient transfer pathway in lieu of a vascular system. The efficacy of these scaffolds was judged on fibroblast distribution and population after 7-12 days of culture.

Keywords: alginate; fibrin; PCL; scaffold; tissue engineering

Introduction

Tissue Engineering is a field of bioengineering that aims to generate functional tissue in vitro or induce regeneration of native tissue in vivo, with the latter often being referred to as Regenerative Medicine. The use of biomaterial scaffolds that facilitate cell growth in a specific 3-D structure is a hallmark of tissue engineering and regenerative medicine. When seeded with cells in vitro, a tissue engineered scaffold should provide an environment conducive to cell growth, where cells can readily attach, migrate, proliferate, and generate endogenous extra-cellular matrix. Following in vitro culture, the resulting tissue construct may be implanted and will hopefully integrate with the patient's native tissue. In regenerative medicine, a scaffold, with or without therapeutic cell populations, may be implanted into a patient's tissue defect with the goal of providing the necessary cues to stimulate natural repair processes, i.e. regeneration of pre-existing nerves in spinal cord injury. In either case, the scaffold must present the cell with an environment in which

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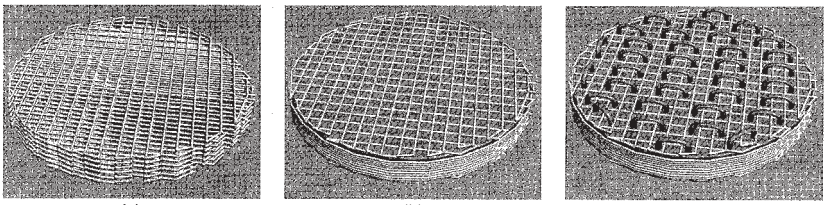
it can thrive in performing the desired tissue-specific function. General criteria to this end include structure of sufficient mechanical strength to maintain construct shape prior to cellular remodeling, nutrient transfer characteristics usually achieved through high porosity, and surface characteristics suitable for cell attachment, growth, and induction of proper differentiation.[1]

Our approach was to construct three-material scaffolds consisting of specialist materials poly-ϵ-caprolactone(PCL), fibrin, and alginate hydrogel in order to meet all of the criteria mentioned. PCL is a biocompatible material with rigidity, mediocre cell attachment, and very long-term degradation capable of being extruded in 200-micron wide struts through precision extrusion deposition.[2] By contrast, fibrin has excellent cell attachment characteristics, is short-term biodegradable, and has comparably poor rigidity.[3] The final material, alginate, has poor mechanical properties, allows no cell attachment when unmodified, and readily diffuses small molecules, valuable for maintaining gas transport.[4] When used in combination, each material has adjustable parameters allowing for ready modification for a variety of applications.

Scaffold Design

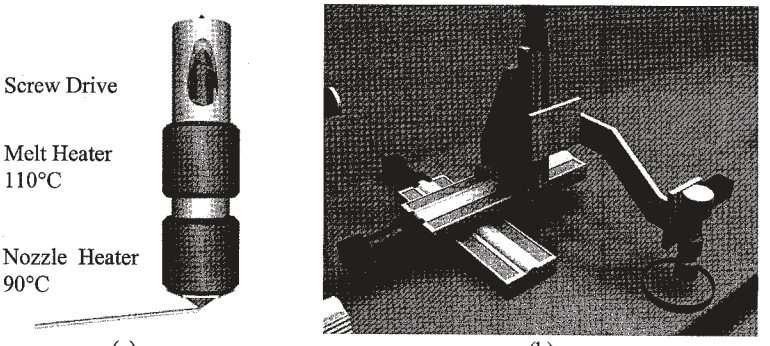
Several experimental series were performed to judge the efficacy of the scaffolds and individual scaffold components through cell culture. The 3-material scaffolds were constructed using precision extrusion deposited PCL as the structural material, alginate thread as a diffusion network, and fibrin to fill the volume. Specimens were created of PCL-only cylinders, PCL+fibrin composite cylinders, and PCL+fibrin+alginate 3-material cylinders. Figure 1 displays the basic construction of (a) a framework PCL-only structure, (b) the same structure with the porespace filled with fibrin, and (c) a three material scaffold with the PCL-fibrin hybrid laced with alginate thread to increase nutrient transfer. Fibrin-only cylinders were also cast to determine the degradation rate for this type of fibrin, derived from bovine plasma fibrinogen, in the context of these cell culture experiments.



(a) (b) (c)

Figure 1. The design of 3-material scaffolds: (a) the PCL structural component, (b) the PCL structural component with pores filled with fibrin, and (c) the alginate diffusion network threaded through the hybrid scaffold.

PCL is a rigid biologically compatible polymer (E = 400 MPa) with mechanical properties comparable to other rigid polymers commonly used in tissue engineering such as poly-lactic-co-glycolic acid (PLGA) (E = 1200 MPa). While not so rigid as naturally occurring structural materials such as hydroxyapatite (E = 14,000 MPa), PCL is exceptionally rigid in comparison to hydrogels such as fibrin and alginate.[5] In addition to structural properties, PCL possesses well-characterized non-toxic degradation, and a slow degradation rate on the order of years. PCL is experiencing a surge in research involving its use in fused deposition. Its low melting temperature of 60° Celsius enables melting and forced extrusion through small caliber nozzles. Precision Extrusion Deposition is a type of fused deposition whereby pellets of polymer are forced through a microscale nozzle through the force of a rotating screw. This extrusion head is mounted on a 3D positioning system allowing construction of complex 3-D structures (Figure 2). The authors have tested a precision extrusion deposition nozzle for PCL with nozzle sizes down to 7 mil.



(a) (b)

Figure 2. The precision extrusion deposition system consisting of (a) the extrusion head mounted upon (b) the 3-D position system.

Initial experiments were conducted into the viability of PCL as a structural component. It was found that precision extruded scaffolds could reliably conform to a variety of porosities and architectures while retaining near-complete porous interconnectivity. Some examples of the scaffolds constructed by this approach are displayed in Figure 3. These scaffolds possess pore sizes of 100 microns (upper left), 450 microns (upper right), 800 microns (lower left), and 100 microns interspersed with 2mm flow channels (lower right). Each scaffold is 21.5 mm in diameter. Micro-CT analysis of these constructs have revealed the capacity to construct tissue scaffolds with porosities ranging from 42-78% porosity with greater than 99.9% pore interconnectivity while retaining a rigid shape.[6]

Fibrin, the component most conducive to cell attachment, is a biologically active naturally derived biomaterial.[4] Fibrin is the dominant structural element in blood clots, and is formed by fibrinogen monomers induced to polymerize in the presence of thrombin and Factor XIII.[7] Sterile fibrin medical products abound, the most common being fibrin glues such as TISSEEL which can be used to seal small wounds during surgery to the point where fluid will no longer pass.[8] Not only is fibrin biocompatible, but it may be derived from mammalian blood. As such, a patient may be treated with fibrin that is 100% native to his body.[3] A wide variety of cells readily attach to fibrin, and, perhaps due to fibrin's natural role in healing, cells attached to fibrin produce a disproportionate amount of extracellular matrix (ECM) when compared to other biologically derived materials such as collagen.[9]

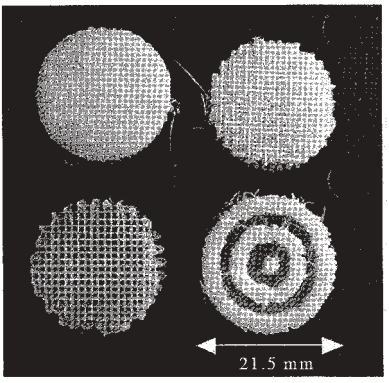


Figure 3. PCL scaffolds of varying pore size, 100 microns (upper left), 450 microns (upper right), 800 microns (lower left), and 100 microns with 2 mm flow channels (lower right).

Cells may be embedded in fibrin readily during the polymerization process. For PCL+fibrin and PCL+fibrin+alginate scaffolds, this is done by mixing equal parts fibrinogen and cell-bearing medium, pipetting the liquid solution into the PCL scaffold and adding a small quantity of thrombin binder. The resulting gel conforms to the available volume available and fills the pores of the PCL scaffold.

Alginate is a biocompatible block co-polymer derived from seaweed. Alginate, in liquid form usually complexed with sodium, undergoes a reversible gelation when introduced to divalent cations such as Ca++.[10,11] Both alginate and its cross-linking agents are biocompatible, as illustrated by the commercial medical products such as the wound dressing material Kaltostat.[12] Alginate gels have relatively weak mechanical properties, with rapid gelation kinetics.[10] As it is also a hydrogel that readily diffuses small molecules, alginate networks within a scaffold can transport nutrients while mitigating the danger of cell growth occluding pores.

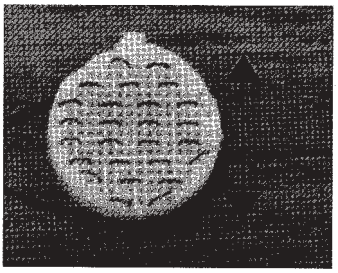


Figure 4. A scaffold with a model of a diffusion network.

The alginate diffusion network is added to the PCL scaffold by gelling a strand of alginate, dehydrating it, and manually threading it through the vertical pores of the scaffold. The gelation of the alginate strand is accomplished by extruding 2% sodium alginate solution through a 250 micron syringe tip into a bath of 5% calcium chloride. The resultant strand is approximately 400 microns in width. It is allowed to dehydrate, reducing its diameter to approximately 200 microns but dramatically increasing its strength. The dehydrated alginate thread is then threaded manually through the vertical pores of the PCL scaffold with a sewing needle. While the alginate itself is transparent, an image of the threading pattern using black polyester thread is displayed in Figure 4. The scaffold in the image is

21.5 mm in diameter. Once rehydrated in medium, the alginate strands return to their 400-micron width, their combined volume representing 2.9% of the available porespace. This does not represent an optimized percentage or geometry, early analytical modeling showing that optimal parameters will vary by cell type specific nutrient consumption, the diffusion coefficient of the desired critical nutrient for transport, and the depth of the scaffold.

The concept of an alginate diffusion network is novel and still undergoing cell culture and analytical validation. It is based upon the premise that nutrient transport is dependent upon the diffusion coefficients of the scaffold. Intuitively, it is to be expected that channels of material with high diffusion coefficients through a second material with low coefficients will increase the total diffusion into the structure. This is similar to a design premise in which large empty channels are included in a scaffold to improve nutrient transfer, a design that has been shown to allow increased viable cell growth deep in a scaffold.[13] Secondly, as time goes on, the diffusion coefficients of alginate will not reduce at the same rate as those of fibrin. As fibrin is degraded by growing cells, it is replaced with ECM and cells themselves, causing pore occlusion and reduction of effective diffusion coefficients.[9] Occlusion due to cell growth is thought to be one of the reasons behind necrotic centers, the regions at a core of a large scaffold where cell death occurs while cells thrive at the scaffold periphery.[13] Alginate, being resistant to mammalian cell attachment, will not suffer this occlusion, and an alginate diffusion pathway to the interior of a scaffold will remain open over time.

Cell Culture

Cell culture experiments were performed with human fibroblasts, growing cells on each scaffold type, PCL-only, PCL+fibrin, PCL+fibrin+alginate to determine the functionality of each component. By necessity, the experiments were broken up into two series, the first with PCL-only scaffolds, and the second with PCL+fibrin and PCL+fibrin+alginate scaffolds. The presence of fibrin fundamentally changed the way cells were seeded, providing 100% seeding efficiency when cells were embedded in fibrin while scaffolds without fibrin had no greater than 40% seeding efficiency. Also, the presence of fibrin and alginate modified the diffusion characteristics sufficiently to make alamarBlue

cytofluorimetry, the preferred method of counting cells on the PCL-only scaffolds, impossible on the hybrid scaffolds.

In the first experimental series, human fibroblasts were grown upon bare PCL-only scaffolds of multiple porosities for 7 days. In all cases, the cells expanded across the surface area of the scaffolds, although there was greater population size and depth of penetration in the scaffolds with the greater pore sizes. Nuclear and lectin stains are displayed in Figure 5. While the PCL-only scaffold appears to be biocompatible, the cells were limited to the surface area of the scaffold struts and never grew across the porespace. This pattern was consistent across the range of PCL scaffold porosities displayed in Figure 3. Cell counts improved with increased pore size, demonstrating differences in nutrient transfer due to porosity.[6]

First order growth kinetics were observed, suggesting that there was a limiting nutrient for cell growth. The distribution of the cells, higher at the surface than at depth despite the influence of gravity implies that the limiting nutrient exists at a concentration gradient, greater in concentration towards the surface. In the PCL-only scaffolds, there should be no vertical concentration gradient of larger molecules such as glucose, which are supplied uniformly every other day. Therefore, it is logical to assume that the limiting diffused nutrient is related to the gas-exchange surface, the surface of the medium. As there was no significant change in pH during culture, carbon dioxide excess cannot be the limiting factor. Therefore, we conclude that the limiting nutrient is oxygen for which there is precedent. In the engineering of bioreactors, oxygen is usually the limiting nutrient for the same reason observed in the scaffold; while other nutrients may be provided uniformly through the medium, oxygen concentration will always be limited by the gas-exchange surface area and conditions.[14,15] When oxygen transfer is the limiting factor in a bioreactor, cell growth and product formation become a function of the mass transfer coefficient, and cells will tend towards first order growth kinetics.[16] Oxygen as a limiting nutrient based on depth is also accepted in basic cell culture technique. It is standard practice to plate cell monolayers no more than 2-5 mm below the surface of medium in cell culture.[17]

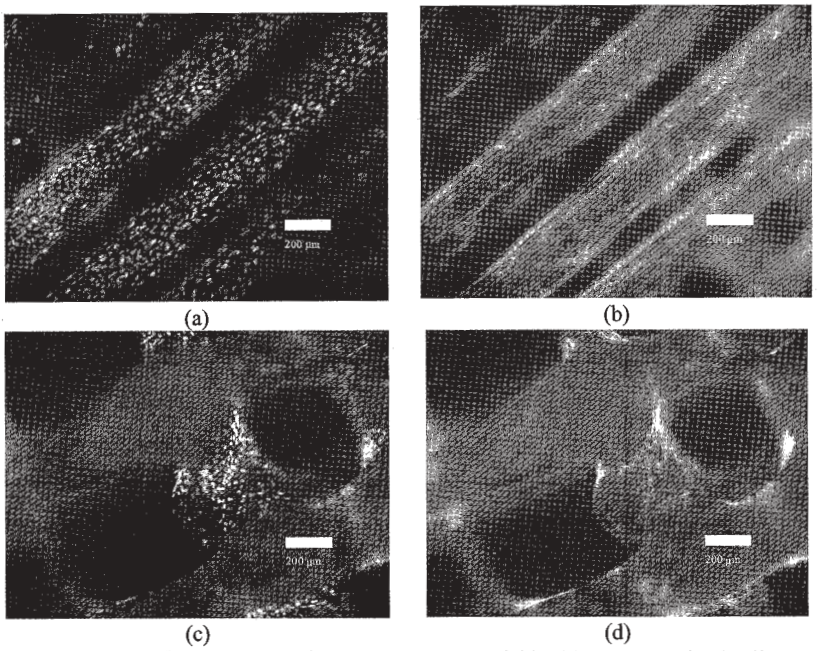
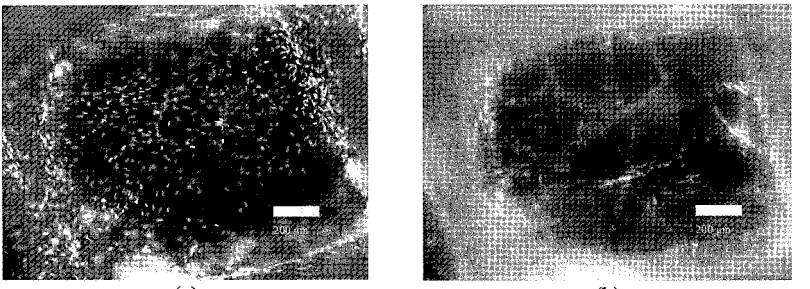


Figure 5. Human fibroblasts growing on bare PCL scaffolds: (a) nuclear stained cells on a 100 micron pore scaffold, (b) lectin stained cells on a 100 micron pore scaffold, (c) nuclear stained cells on a 450 micron pore scaffold, and (d) lectin stained cells on a 450 micron pore scaffold. In all samples, the cells grew uniformly on the surface of the PCL struts but did not cross any of the intervening pore channels. (bar = 200μm)

A second experimental series was performed with the PCL+fibrin and PCL+fibrin+alginate scaffolds under fibroblast culture out to 12 days, to determine if fibrin filler improved cell distribution across pores and if an alginate diffusion network increased nutrient transfer to the depth of the scaffolds. The additional time, compared with the 7 day PCL-only experiments, was allowed to both permit the fibrin to degrade and to allow for larger cell populations, larger cell populations increasing the visibility of nutrient transfer limitations. In this case, all scaffolds possessed uniform pore sizes of 1000 microns, to minimize the effect on nutrient transfer of the PCL component.

The presence of fibrin as a filler allowed the cells to cross the pore spaces and replace the existing fibrin with their own ECM. Figure 6 displays a pair of images taken from a PCL+fibrin scaffold after 12 days of culture. The vertical pore seen in each image is 1000 microns across, with the cells completely crossing the square porespace at center. As this

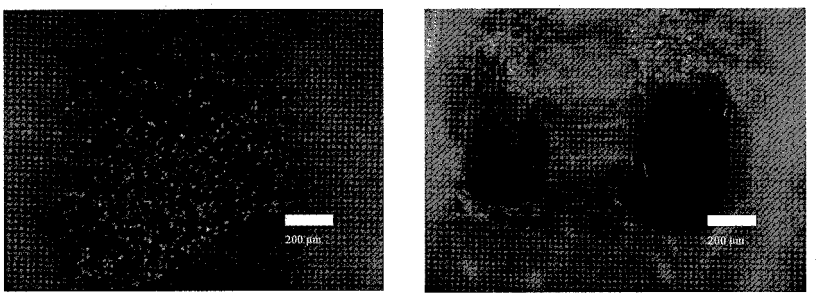
image was taken at 12 days, most of the fibrin has already degraded, and the matrix that supports the fibroblasts consists of secreted ECM. Images from the sample at depth, as in Figure 6b, show 3-D networks of fibroblasts crossing the pore, in effect building their own scaffold for further growth. While qualitatively there was a great deal of ECM present, reminiscent of fibrous tissue within the body, it was at highest concentrations at the top of the scaffold, where oxygen was readily obtainable.



(a) (b)

Figure 6. Nuclear stained human fibroblasts grown on a PCL+fibrin scaffold. The image at left (a) is the surface of a single vertical pore, and (b) is the same pore at .25 cm depth. Unlike the PCL-only scaffolds, in the PCL+fibrin scaffolds, the cells were able to grow across intervening pore channels, although not uniformly. At depth in a vertical pore, 3D networks of fibroblasts were observed. (bar = 200μm)

Scaffolds possessing all three materials demonstrated a different distribution of cell growth. As shown in figure 7, scaffolds possessing a diffusion network generated a denser pattern of cell growth at the surface of each pore. In Figure 7a, the square pore in focus is completely filled with fibroblasts with a thin film of ECM generated on top of the scaffold. By contrast, the cells upon PCL+fibrin scaffolds had a more 3D topography, with most pores possessing pits approximately 500 microns in depth. In addition, cross sections revealed cells in the scaffolds with alginate diffusion networks grew at all depths within the scaffold while cells in the scaffolds lacking the diffusion networks only grew in the upper half of the 5 mm scaffolds. This tentatively supports the theory that alginate diffusion networks increase oxygen diffusion from the surface to depth.



(a) (b)

Figure 7. Fibroblast growth after 12 days on PCL+fibrin+alginate scaffolds (a) and PCL+fibrin scaffolds (b). Comparison of the surface cell growth in vertical pores of both scaffolds showed more uniform cell growth across the pores of PCL+fibrin+alginate scaffolds. PCL+fibrin scaffold vertical pores were seen to have pits and 3D topography at the surface. (bar = 200μm)

Further experimentation is required to verify the function of the diffusion network and determine the critical depths to which diffusion networks may function. This will be done by creating multi-level scaffolds in which cell counts may systematically be measured optically at all depths of the scaffold. Multiple cell types will be utilized in this experimental series to measure the differences of cell parameters such as growth rate, maintenance coefficient, and oxygen consumption rate on critical depth. This experimental series will coincide with the development of a computational model to predict oxygen and cell distribution with different diffusion network geometries.

Conclusion

Preliminary experiments with hybrid scaffolds have illustrated their capacity to be built to desired shape and porosity and to provide a cell attachment substrate. The use of a diffusion network appears to enhance oxygen delivery to the depths of a scaffold, as interpreted from more robust cell growth on scaffold surfaces and deeper cell penetration. Future experiments will test other cell types on hybrid scaffolds while varying their component parameters. Chief among this work will be a complete analytical model of the function of the diffusion network dependent upon geometry, material properties, and cell oxygen consumption demands. By modifying parameters of each individual scaffold component on a very local level, it is hoped that tissue interfaces may be constructed, allowing specific cell types to thrive in specific regions of the same scaffold.

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