

Mechanical Testing and Optimizing Cell Seeding on Porous Fibrin Scaffolds

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Abstract: *There have been numerous studies on engineering an artificial esophagus and esophageal substitutes in the recent years. They include autologous tissues, artificial materials (Teflon and polypropylene), and porous scaffolds and meshes using polyvinylidene fluoride (PVDF), collagen, and decellularized matrices (i.e., acellular porcine). However, complications such as stenosis, leakage, and fibrosis, often occur. We present here a tissue engineering approach to creating an esophageal replacement using cell-seeded fibrin scaffolds as in vitro models of engineered tissues. Templated fibrin scaffolds were found to have initial mechanical properties independent of pore size. Various cell seeding techniques to distribute cells throughout the interconnected microporous fibrin scaffolds were examined. Although we found that standard cell seeding procedures are inadequate in distributing cells deeply into the scaffolds, the scaffolds were shown to support cell adhesion and growth. Biodegradable fibrin scaffolds demonstrate potential as a future tissue engineered scaffold for esophageal replacements.*

1. INTRODUCTION

Esophageal cancer is not as common as other cancers such as lung, breast, or prostate cancers, but it is on the rise. There are approximately 15,000 new cases of esophageal cancer annually in the U.S. In 1996, 11,000 deaths were attributable to the disease [5]. Esophageal cancer accounts for 5% of gastrointestinal cancers and 1% of new cancers in the U.S. [1]. Current treatments of esophageal cancer include chemotherapy, radiotherapy, esophagectomy, and any combination of these three. The most common form of therapy is partial or complete esophagectomy, however, certain treatments are more suitable than others depending on the stage of the cancer. Despite the variety of approaches to treatment, the success rate is quite disappointing because the cancer is typically diagnosed at a relatively late stage. Patients who receive treatments can expect to live and be cancer-free for only a short duration of about five years [5]. Moreover, these treatments can cause additional problems such as leakage, stricture, and abnormal elongation of the implanted tissue propose a bad quality of life for patients. Therefore, new therapies that can improve patient quality of life after treatment for esophageal cancer are needed.

In response to this need for alternative therapies, a large number of studies on engineered artificial esophagus and esophageal substitutes have been conducted in recent years. Artificial materials such as Teflon® and polypropylene have also been used in clinical

trials, but they often lead to development of fibrosis and leakage [13]. Autologous tissues such as gastric fundus, ileal pedicle, pleura, skin, and intercostal muscle have all been tested in humans [4,13]. However, all of these materials lead to complications including stenosis and leakage. Lastly, protein based scaffolds such as collagen and decellularized matrices (i.e., acellular porcine) have been constructed [10,15]. Although the protein based scaffolds led to subsequent regeneration of tissues, stenosis was still a major failure mechanism of the materials [15].

Optimal pore sizes have been determined for a number of tissue engineering applications. In bone tissue engineering, larger pores in the range of 300 to 400 μm have been shown to enhance osteoblast proliferation and mineralized tissue production [14]. However, for angiogenesis to occur, smaller pores on the order of 30 μm are optimal [12]. Cells not only respond to a scaffolds pore size, but also to its mechanical properties [3]. Therefore, in developing a system to evenly compare scaffolds of different pore sizes it is critical to evaluate the effects of pore sizes on mechanical properties.

A common strategy for the engineering of tissues involves the seeding of cells onto porous scaffolds. In many studies, seeding prior to implantation has been shown to result in regeneration of complex structures with characteristic cell lining [2,9]. For example, in an arterial conduit model by Kaushal *et al.*,

scaffolds that were preseeded with cells exhibited extensive vessel-like properties such as contractile activity, but not when implanted as an acellular control scaffold [11]. Currently, several seeding methods are used, including simple techniques such as static seeding where cells and scaffolds are simply brought into contact and allowed to sit, to more elaborate techniques such as pulsatile perfusion where culture medium and cells are pulsed through the scaffold constantly under oscillatory flow controls to mimic the physiological conditions. Active seeding has been shown to improve efficiency, but great variation is seen from model to model [6].

For cell seeding to be effective, the scaffold must be able to allow cells to adhere, grow, and proliferate. Fibrin has been studied extensively to promote cell adhesion and increased protein synthesis [7,8]. Moreover, various scaffold modifications were able to enhance cell affinity of porous scaffolds and subsequent cell seeding [16].

The objectives of this work were to measure fibrin scaffolds tensile strengths to examine the effect of pore sizes on mechanical parameters and to optimize cell seeding of scaffolds by examining several standard cell seeding methods.

2. MATERIALS AND METHODS

2.1 Fabrication of fibrin scaffolds

Porous fibrin scaffolds (5 mm diameter and 0.7 mm thickness) with four different ranges of pore size (30-38, 38-50, 50-58, and 75-95 μm) were constructed by the 6 S method of sieve, shake, sinter, surround, solidify, and solubilize. First, poly(methyl methacrylate) (PMMA) beads were sieved on a ATM sonic sifter to form groups of beads having homogeneous diameters. The sieving consisted of two passes; rough pass for 20 min x 3 iterations and fine pass for 5 min x 5 iterations. Next, they were put in a Teflon mold placed between two glass slides (Figure 1), shaken using an Aquasonicator for 20 min to form a close pack of beads, and sintered at 145°C for 22 hrs to produce 50% neck size. The scaffolds were next covered with a fibrinogen

monomer solution (200 mg/ml in 0.9% NaCl) and the air surrounding the beads was removed under a vacuum of 30 mmHg and released to draw in the fibrinogen solution. Next, the fibrinogen monomers were polymerized to fibrin with a thrombin solution (267 units/ml Thrombin(Sigma) and 110 mg/ml 1N CaCl_2 into 18.87 ml Dulbecco's Modified Eagle's Medium (DMEM)). Finally, the beads were solubilized in 100% HPLC grade acetone (3 x 4 hrs) leaving behind a microporous fibrin scaffold. The scaffolds were then rehydrated through a series of graded ethanols into a phosphate buffered saline (PBS) solution.

2.2 Mechanical testing

Tensile tests were conducted on 5543 series Instron® mechanical tester with pneumatic grips and a 10 N load cell. Scaffolds were trimmed to 5 mm x 20 mm x 1.2 mm for testing and loaded with a grip pressure of 50 lbs/sq. inch. Samples were precycled for three cycles at a rate of 10 mm/min to 25% strain before the data collection took place. They were then strained at a rate of 10 mm/min until failure. The data were analyzed using Instron Bluehill® software v. 2.5. Young modulus was calculated by fitting a linear line on a stress-strain curve at the initial strain region which occurred at 20-40% strain following precycling.

2.3 Cell culture conditions

NIH 3T3 fibroblasts were cultured in 75-cm² tissue cultured flasks (Falcon Labware, Oxnard, CA) using supplemented culture medium (DMEM, 15% fetal bovine serum (FBS), and 1% antibiotic/antimycotic (Sigma)). Cells were incubated in a humidified incubator buffered with 5% CO_2 .

2.4 Scaffold preparation for seeding experiments

Scaffolds were sterilized in 6-well plates by transition through graded ethanol, sterile PBS, and finally into supplemented culture medium. They were then transferred into 24-well plates where they were the same size as the wells and topped with sterile cloning rings.

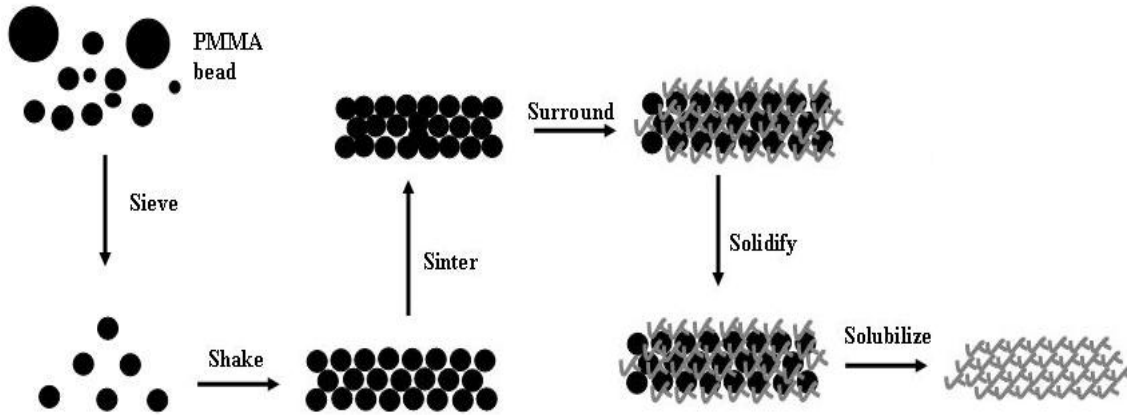


Figure 1 Summary of the 6-S fabrication method. The method involves sieving, shaking, sintering PMMA beads, surrounding the beads with fibrinogen monomer solution, solidifying the fibrinogen monomer to make fibrin polymers, and solubilizing the PMMA beads leaving only a micro-porous fibrin scaffold.

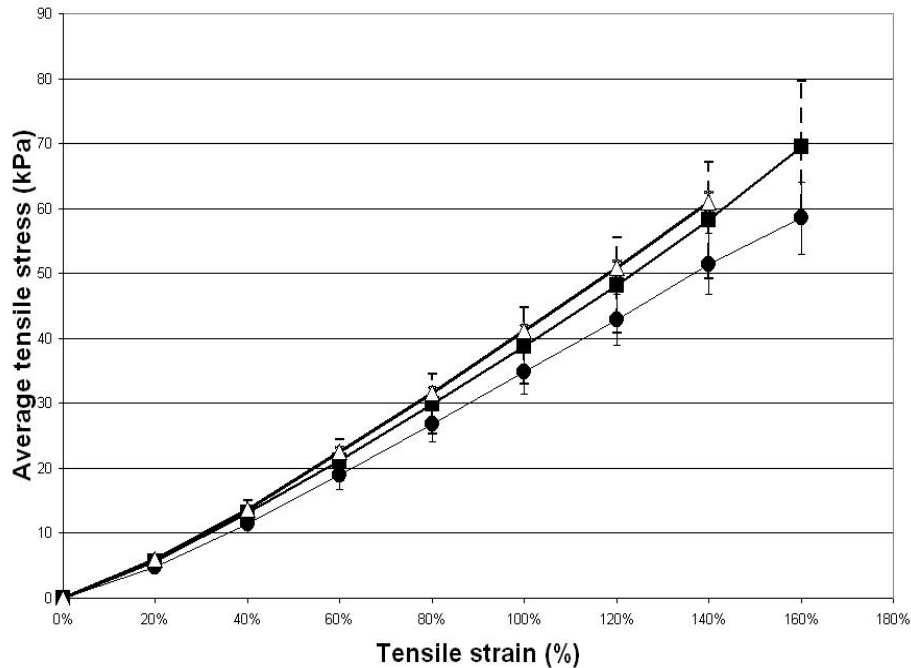


Figure 2 Average stress-strain curves of tension test result of fibrin scaffolds with various pore sizes. Each group of samples with same pore size is represented by a curve. The first curve that connects the circles represents the stress-strain curve of the 30-38 pore sized samples. The second curve that connects the squares represents the stress-strain curve of the 50-58 pore sized samples. Finally, the last curve that connects the triangles represents the stress-strain curve of the 75-95 pore sized samples.

2.5 Cell seeding methods

Fibroblasts were harvested using versene + 0.01% Trypsin. Cells were collected and diluted to 5 million cells/ml. 200 μ l of cell mixture was delivered to each scaffold. The scaffolds were divided into three groups that received different

seeding treatments: centrifuge, orbital shaker, and combined centrifuge/orbital shaker seeding. In the centrifuge method, cell-seeded scaffolds were centrifuged for 5 min at 1000 rpm. The scaffolds were then incubated in a humidified incubator for 4 hrs to allow for attachment

before being fixed. For the orbital shaker method, cell-seeded scaffolds were placed on an orbital shaker at 60 rpm in a humidified incubator for 4 hrs prior to fixing. Lastly, in the combined centrifuge and orbital shaker method, cell-seeded scaffolds were centrifuged for 5 min at 1000 rpm, then placed on an orbital shaker at 60 rpm in a humidified incubator for 4 hrs prior to fixing. All scaffolds were fixed in methyl carnoys overnight.

2.6 Staining and imaging

Fixed scaffolds were embedded in paraffin and 5 μm sections were taken at depths of 100, 200, 300, 400, and 500 μm. The slides were stained with hematoxylin and eosin (H & E), and then imaged on a Nikon E800 upright microscope with 4x and 10x objective lens to view cells.

Cells in representative views were counted by their nucleus.

3. RESULTS

3.1 Mechanical testing

There was no variance of mechanical properties between scaffolds that had different pore sizes; therefore, pore sizes did not have any significant effect on mechanical properties of fibrin scaffolds. The Young’s modulus for scaffolds with pore sizes between 30 and 38 μm was found to be 38.3 kPa. Similarly, 50-58 μm and 75-95 μm pore scaffolds were found to be 37.1 and 39.0 kPa, respectively. Figure 2 shows all stress-strain curves were located within a small region on the plot. Table 1 shows that tensile Young’s modulus of fibrin scaffolds was essentially the same between scaffolds.

Pore size (μm)	30-38	50-58	75-95
Young Modulus (kPa)	38.385	37.156	39.013
Standard deviation	+/- 4.124	+/- 6.280	+/- 1.137

Table 1 Tensile Young’s modulus of fibrin scaffolds with various pore sizes. The Young modulus values of all samples are close among themselves.

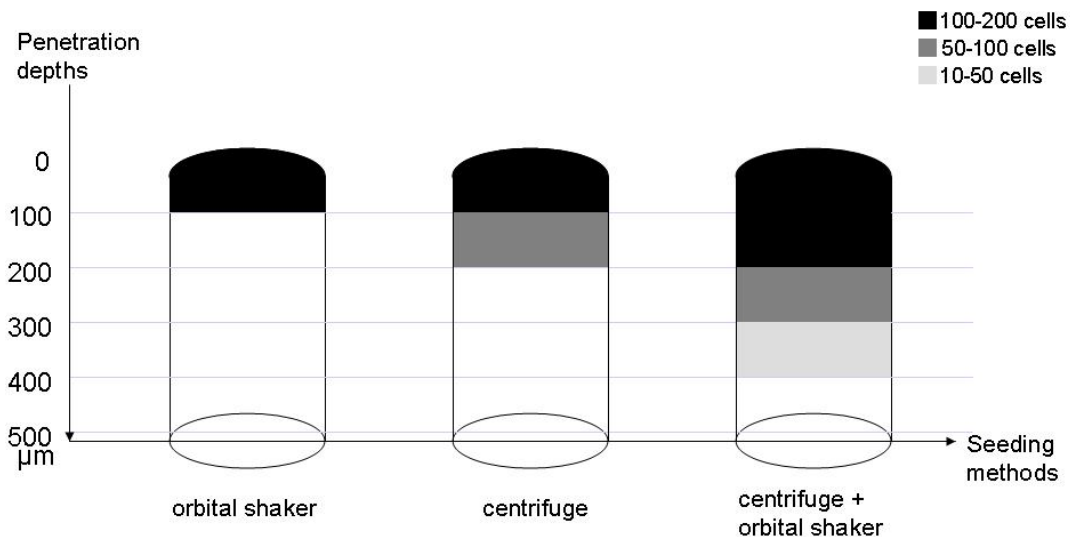


Figure 3 Summary of the number of cells on each view planes of seeded scaffolds that were seeded with various seeding techniques at various depths. All scaffolds had pore size of 50-58 μm. The number of cells was collected visually on random plane view and categorized into depth levels.

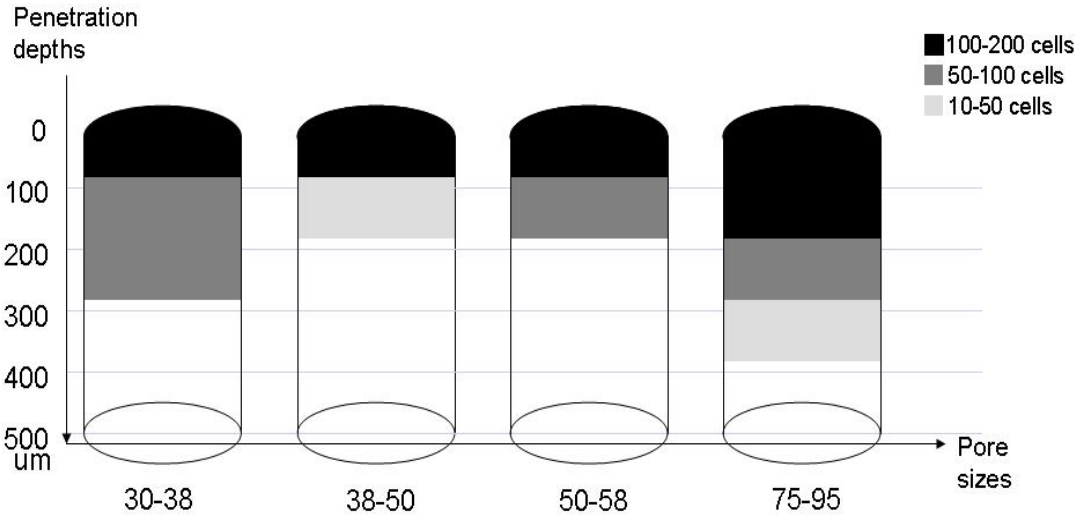


Figure 4 Summary of number of cells on each view planes of different pore-sized scaffolds that were seeded with the centrifuge method at various depths. The number of cells was collected visually on random plane view and categorized into depth levels.

3.2 Cell seeding experiments

Figure 3 summarizes the distribution of cells found at various depths throughout 50-58 μm scaffolds that were seeded with the three seeding methods. The orbital shaker method had many cells on top of the scaffolds (100 μm depth) but not so many inside the scaffolds. The centrifuge method had cells down to 200 μm depth. Lastly, the combined method had cells down to 400 μm

depth. The number of cells decreased at deeper depths.

Furthermore, Figure 4 summarizes the distribution of cells found in various pore-sized scaffolds that were seeded with the centrifuge method. Larger pore-sized (75-95 μm) scaffolds generally had larger number of cells in them at deeper depths compared to scaffolds with

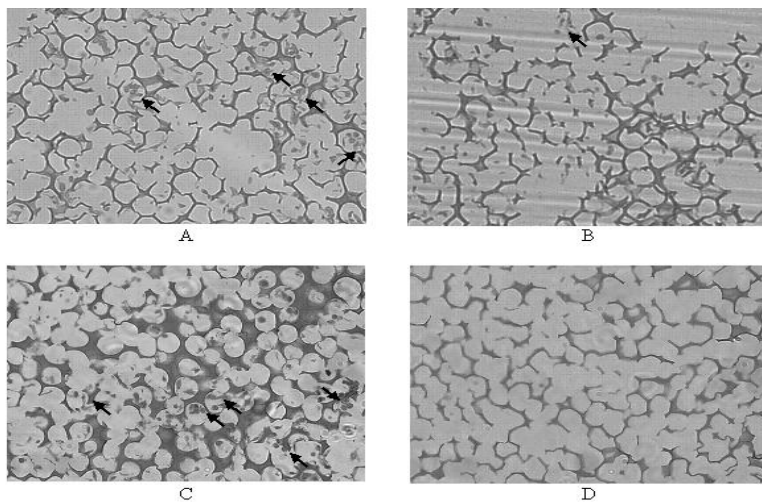


Figure 5 H&E image of seeded-scaffold after four days in culture (10x magnification). The arrows show representative areas of which cells are highly concentrated. A and B are images that are taken from scaffold which has seeded with cells using the combined centrifuge and orbital shaker method. C and D are images that are taken from scaffold which seeded with cells using the centrifuge method. A and C are sections at 100 μm deep; B and D are at 300 μm deep.

smaller pore size. Figure 5 contains H&E images of 50-58 scaffolds after four days in culture (10x objective). A and B are images that were taken from scaffolds which were seeded with cells using the combined centrifuge and orbital shaker method. C and D were images that are taken from scaffold which seeded with cells using the centrifuge method. A and C were sections at 100 μm deep; B and D were at 300 μm deep. It can be seen that there are more cells in A than in B-D.

4. CONCLUSION

We have attained a general knowledge on mechanical properties of a templated fibrin scaffold. Pore size is not a determining factor of the mechanical strength as predicted by Yunoki *et al.* [17]. The combined centrifuge and orbital shaker method is the best standard seeding method, and larger pore size of enhances cell seeding significantly. The centrifuge method was superior to the orbital shaker at delivering cells to deeper depths. Moreover, scaffolds with larger pore size (75-95 μm) had a larger number of cells in them at deeper depths compared to scaffolds with smaller pore size; therefore, scaffolds with large pore size (75-95 μm) were better for cell seeding.

Although cells were found in scaffolds, the numbers of cells were not as large as expected. We found that standard cell seeding procedures are inadequate in distributing cells deeply into the scaffold; however, the scaffold will support cell adhesion and growth and thus the scaffold could be a promising matrix in tissue engineering. Future studies should explore other modified seeding methods, scaffold fabrication techniques, and *in-vivo* examination of functions and interactions of cell-seeded scaffold in tissue repair.

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