Structural Characterization of Engineered Muscular Mimetic

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ABSTRACT

An engineered muscular mimetic (EMM) was synthesized, and its overall structure—porosity, average pore size, and pore alignment—was characterized and compared to actual decellularized skeletal muscle (DSM). The EMM’s porosity was about 20% more and average pore size was about 85% smaller compared to that of the DSM. Unlike the strong horizontal pore alignment in the DSM, no distinct orientation could be identified in the EMM structure.

1. INTRODUCTION

Growing interest in tissue regeneration has sparked a realization of the clinical significance successful cell transplantation could have on many patients. Despite the ongoing research and progress that has been made in tissue engineering, difficulties still arise in making biocompatible engineered tissues, which integrate and grow within the cell matrix already present in the body. Also, production of engineered tissue with structures that mimic native tissues has been an issue (Griffith & Naughton, 2002).

Ongoing research and recent advance in tissue engineering techniques, however, has allowed the development of many promising solutions. For example, in an effort to circumvent the foreign body response (FBR) that is often initiated after implementation of ‘tissue engineered’ (either synthetic or degradable) polymer based scaffolding, researchers at the University of Utah have suggested using biological scaffolds made of extracellular matrix (Wolchok & Tresco, 2010). In their study, they outline the methodology used to develop not only a extracellular matrix (ECM) composed of entirely naturally derived biomaterials but also to culture the ECM in larger quantities than previously possible. Their general approach involved seeding and then culturing human derived fibroblasts onto open cell foams, after which they used a water miscible solvent to remove the synthetic foam material. Characterization of the remaining ECM was conducted and revealed that “when the extracted material [i.e. the ECM] was reseeded with human derived fibroblasts, cells attached, spread, and remained viable” (Wolchok & Tresco, 2010).

The primary goal for my study was to synthesize engineered muscular mimetic (EMM)—using similar methods as the researchers at the University of Utah—and characterize its structure by analyzing the porosity, average pore size, and pore alignment of the EMM. For comparison, decellularized skeletal muscle (DSM) taken from the lateral gastrocnemius of a rat was also characterized.

Characterization of EMM and DSM was done primarily by using software called ImageJ. First, histology images of both EMM and DSM were taken with a light microscope (400x total magnification) and dissection scope (20x total magnification). Using ImageJ, the porosity was found by measuring what percentage of a certain area did not contain decellularized material. To find the average pore size, the average area of randomly selected pores was measured. To calculate the pore alignment, a Fast Fourier Transform (FFT) algorithm was first run on the image. Summing the radial intensities (at some angle θ), the contribution of the object in that direction could be quantified (Alexander, Fuss, & Colello, 2006). In this way, the overall directional content of the EMM and DSM could be realized.
2. METHODS

2.1 Preparation of cell derived materials

The polymer foams were fabricated using medical grade polyurethane elastomer (Tecoflex SG-80A, Thermedics). Polyurethane pellets were dissolved in dimethylacetamide (DMAC) (10% solution by weight) in a round bottom flask under a fume hood. The solution was heated at 60°C and stirred continuously overnight.

To prepare the sugar scaffolds, 200uL of DI water was added drop-wise to 10g of sugar. The sugar and DI water were mixed using a spatula. The wetted sugar was placed into four 1-inch molds, making sure that it was loosely packed and the top was flat and level. The sugar molds were then placed in an oven at 50°C overnight to dry.

Once the sugar molds were dry, the DMAC/SG-80A solution was added drop-wise to it, so that the sugar was saturated but no excess ‘puddles’ were formed. About 2ml of DMAC/SG-80A solution was used per mold. If too much solution was added and the sugar molds were oversaturated, sugar was lightly ‘dusted’ on top. These precautions were taken so that the porous integrity of the polymer scaffold would remain throughout the foam and a non-porous polyurethane layer would not form on top. Then, the molds were submerged in a DI water bath. After 24 hours, each polymer scaffold was carefully removed from the mold using tweezers.

Foams that had formed a thin layer of polymer on top were discarded. The foams were placed in a fresh DI bath with a stirrer (600rpm). After 3 hours, the DI water was discarded and replaced with fresh DI. The foams were rinsed this way several times.

Prior to seeding the foam scaffolds, rat myoblast cells (L6, ATCC, Manassas, VA) in their fourth passage were thawed, plated onto several T-175 flasks, and grown to confluency in DMEM with 10% fetal bovine serum (FBS) and 1% Glutamax. Confluent flasks were dissociated with .25% trypsin and DMEM, centrifuged, and resuspended in DMEM. The cells were counted with a hemocytometer and seeded onto the foams at a density of 5 million cells per foam. The cell seeded foams were maintained for four weeks. Media was changed every 2-3 days, and cells were supplemented with TGF-Beta1 (4ng/ml) and 10% ascorbic acid.

To isolate the engineered muscle mimetic (EMM) from the polymer, each foam was removed from the petri dish and placed in individual glass scintillation vials. Then, 10mL of DMAC was slowly added to each bottle. After 45 minutes, DMAC was removed and 5mL of fresh DMAC was added. The EMM was rinsed with DMAC every 24 hours over four days. The DMAC was removed after the last rinse and DI water was added. After 20 minutes, the DI water was removed and replaced with fresh DI; the EMM was rinsed this way several times.

The EMM was then decellularized (Gillies, Smith, Lieber, & Varghese, 2011). EMM was incubated in 50nM Latrunculin B at 37°C with agitation for 2 hours, washed two times with DI water for 15 minutes each, incubated with 0.6M KCl for 2 hours at room temperature, rinsed with DI water again, incubated with 1M KI for 2 hours at room temperature, incubated with DI water at room temperature for 24 hours, incubated with DNAase for 2 hours at room temperature, and finally incubated with DI water overnight at 4°C. The EMM was then submerged in 3mL of fresh DI water, frozen in the -80°C freezer, freeze-dried, and weighed.
To compare the structure of the EMM to native skeletal muscle, decellularized skeletal muscle (DSM) was also prepared. The lateral gastrocnemius muscle of a rat was first dissected. Decellularization of skeletal muscle was conducted similarly to EMM. The skeletal muscle was first incubated in 50nM Latrunculin B at 37°C with agitation for 2 hours and washed two times with DI water for 15 minutes each. Then, the muscle was incubated with 0.6M KCl for 2 hours at room temperature, then 1M KI for 2 hours at room temperature, and finally DI water at room temperature overnight; this was repeated six more times. Then, DSM was incubated in 1ku/mL DNAase for 2 hours and in DI water for 48 hours at 4°C. The DI water was removed from DSM and fresh DI water was added. The DSM was placed in -20°C until frozen, freeze-dried, and weighed.

2.2 Characterization

Small sections of the engineered muscle mimetic (EMM) and decellularized skeletal muscle (DSM) were paraffinized, sectioned with a microtome (5um), and mounted onto microscope slides. Samples were deparaffinized by incubating them twice in toluene for 5 minutes, twice in 100% ethanol for 1 minute, twice in 95% ethanol for 1 minute, and in PBS for 15 minutes. Subsequently, the slides were stained in hematoxylin and eosin phloxine; the slides were incubated in hematoxylin for 5 minutes, rinsed with DI water, incubated in eosin phloxine for 30 seconds, rinsed in DI water, and rinsed in 95% ethanol. Slipcovers were placed on all the slides. Histology images of both EMM and DSM were taken with a light microscope (400x total magnification) and dissection scope (20x total magnification). Using ImageJ, the porosity, average pore size, and pore alignment of the EMM and DSM was analyzed.

Porosity was calculated from images as the percent area not containing either EMM or DSM material. The histology image was first opened in ImageJ, and the “Type” was changed from “RGB Color” to “8-bit.” Then, the contrast in the image was increased, and the threshold was adjusted so that only the ECM was visible (the rest was covered by the dark background). Next, the measurements were set so that they calculated the area and the area fraction within the threshold limits. The percent void area was measured for each histological image; there were nine EMM (400x) and six DSM (20x) images analyzed.

A similar approach was taken to find the average pore size. Like before, the image was changed to “8-bit,” the contrast was increased, and the threshold was adjusted. Then, the “freehand selection” tool was used to outline and measure the area of random pores; there were nine EMM (400x) and six DSM (20x) images analyzed.

To calculate pore alignment, a Fast Fourier Transform (FFT) algorithm was run on the DSM and EMM images (both at 20x). The FFT decomposed the image into its sine and cosine components; its output was an image consisting of pixels whose distribution and intensity was representative of the directional content of the original image. To quantify the directionality of the pores, the pixel intensities were first summed along a straight line from the center to the edge of the image. This quantity indicated how much of the DSM or EMM material was found along that angle. Using ImageJ, the DSM and EMM images were converted from “RGB” to “8-bit”. Then a threshold level was established such that it included all the muscular or engineered
material. Once the threshold level was applied, the resulting image was black and white; the black (which was the color of the muscular or engineered material) had a frequency value of 255 and the white had a value of 0. A FFT was run, and the subsequent image was rotated 90 degrees to the left so that it matched the orientation of the original image. To sum the intensities along various angles, an Oval Profile plugin was used. An oval was drawn on the FFT image; the edges of the oval extended to the edges of the image. An Oval Profile was run with ‘360’ number of points and ‘radial sum’ analysis mode. The Oval Profile then produced a graph that showed the intensities summed along designated angles. To get quantitative and comparable evaluations of the pore alignment, the ratio of horizontally to vertically summed intensities on the FFT was taken. Values greater than 1 indicated a greater horizontal alignment, values less than 1 indicated a greater vertical alignment, and values equal to one indicated irresolute orientation.

3. RESULTS AND DISCUSSION

Following a four-week culture period of L6 rat myoblast in a polyurethane foam scaffold and subsequent decellularization, EMM samples yielded 4.4 ± .7 mg of extractable material for every foam seeded. Thin sections of EMM were fixed onto slides and stained with hematoxylin and eosin. The slides were then examined under 400x total magnification light microscope and 20x total magnification dissection scope (Fig. 1).

![Figure 1: (a) 20x total magnification, dissection scope, EMM image and (b) 400x total magnification, light microscope EMM image](image)

The images revealed an interconnected porous network of what appeared to be accumulated extracellular material. The structure and shape of the EMM resembled the cavities and pores that were present in the foam.

Muscular tissue taken from the lateral gastrocnemius of a rat was decellularized. The DSM samples yielded 210 ± 14 mg of material. Similarly to the EMM, the DSM was fixed onto a slide, stained with hematoxylin and eosin, and analyzed under 400x total magnification light microscope and 20x total magnification dissection scope (Fig. 2).
The images were as expected for decellularized muscular tissue. The DSM showed a striated structure composed of the extracellular material left after decellularization.

### 3.1 Characterization

Using ImageJ, the porosity, average pore size, and pore alignment could be quantified; in each case, there were nine EMM and six DSM analyzed. The porosity was measured as a percent of void area over total area, for the EMM and DSM (Fig 3). The EMM muscle was more porous with an average porosity of $86.4 \pm 5.4\%$; the DSM had an average porosity of $71.7 \pm 4.3\%$.

Next, the average pore size was measured by finding the area of randomly selected pores. Figure 3 shows the average pore size in x1000 μm$^2$. The average pore size of EMM was $15,000 \pm 9600 \mu m^2$ and of DSM was $104,000 \pm 20,000 \mu m^2$. 

### Figure 2: (a) 20x total magnification, dissection scope, DSM image and (b) 400x total magnification, light microscope DSM image

### Figure 3: porosity and average pore size measured for DSM (A) and EMM (B). Average porosity of EMM and DSM was $86.4 \pm 5.4\%$ and $71.7 \pm 4.3\%$, respectively. Average pore size of EMM and DSM was $15,000 \pm 9600 \mu m^2$ and $104,000 \pm 20,000 \mu m^2$, respectively.
An approximate 20% porosity and 85% pore size difference between EMM and DSM indicates that some manipulation of the protocol should be made to obtain a more desirable structure. In the realm of using porous scaffolds to engineer tissues, there are some methods available to obtain more appropriate and controlled porosity and pore size. Tai et al. used supercritical carbon dioxide during foam scaffold processing to create bubbles in the polymer melt as the carbon dioxide escaped. Using supercritical carbon dioxide, they were able to create tailored pore size and structure by manipulating the process conditions: pressure, time, and temperature (Tai, et al., 2007). More traditional techniques using organic solvents and/or elevated temperatures are harder to manipulate and control and are more limited on possible architecture constructs. In some cases, even after manipulation of architecture (i.e. scaffold pore size) and incubation time, a limited and narrow range of cell proliferation and extracellular matrix formation can occur (Lee, Wu, & Dunn, 2007).

Figure 4 shows the original images of the DSM and EMM, then the FFT of that image as well as the radial lines along which the intensities were summed, and finally a graphical representation of the summed intensities at each angle. For the DSM, the graph (Figure 4, a-iii) shows high and broad peaks near 0° or 360° and 180°. This corresponds to a horizontal alignment as can be affirmed by DSM image (Figure 4, a-i). In contrast, the graph for the EMM (Figure 4, b-iii) does not show any broad peaks. Instead it has a fairly uniform distribution of intensity levels. This implies that there is no overriding alignment as supported by ESM image (Figure 9, b-i).

![Figure 4: Analysis of DSM (a) and EMM (b) pore alignment using FFT analysis (a-ii, b-ii). The intensities are summed at each angle (a-iii, b-iii). DSM shows a horizontal alignment (0°, 180°, and 360°). EMM shows no distinct alignment.](image-url)

More quantifiable and comparable values for evaluating the alignment were obtained from taking the ratio of horizontally to vertically summed intensities (Fig 5). As mentioned before, values greater than 1 indicated a greater horizontal alignment, values less than 1 indicated a greater vertical alignment, and values equal to one indicated irresolute orientation. The horizontal to vertical ratio for EMM was 1.00±.05 and for DSM was 1.12±.05. To compare the significance of the alignment ratio calculated for EMM and DSM, a two-sample t-test was conducted. A p-
value of .04 was obtained. Therefore, the alignment ratio for EMM and DSM had a statistically significant difference. These results further support the alignment assessment conducted with the FFT; EMM has a nondescript alignment, while DSM has a strong horizontal alignment.

To obtain the desired horizontal alignment, current methods of scaffold formation cannot be used; it does not allow for any particular orientation. Current technologies are exploring ways to provide appropriate physiological stresses during engineering of a tissue. For example, bioreactors for culturing cells are being designed so that they provide conditions and stresses that are typically seen in vivo during tissue generation, such as shear stresses, compression, and pulsatile flow of culture medium (Griffith & Naughton, 2002). Tanzi et al. used another method that was more effective and that more closely followed the methods used in this study (in principle at least). They used a scaffold made of biodegradable PLLA-TMC (Lactide-trimethylene carbonate) copolymer. The polymer shape mimicked that of muscle tissue and was made by employing two different masters: a modeling past sheet imprinted with fibers and an alumina master made from laser fabrication. The cells grown on the polymer were able to follow the general alignment of the polymer, although their filopodia did extend in a random orientation over the grooves scaffold.

4. CONCLUSION

An engineered muscular mimetic (EMM) was synthesized, and its overall structure—porosity, average pore size, and pore alignment—was characterized and compared to actual decellularized skeletal muscle (DSM). The EMM’s porosity was about 20% more and average pore size was about 85% smaller compared to that of the DSM. Unlike the strong horizontal pore alignment in the DSM, no distinct orientation could be identified in the EMM structure. Although an EMM was successfully produced, there are many strides yet to be made in engineering tissue that more closely mimics the structure of muscle cells. For example, an obvious next step could be to try and form scaffolds with long thin pores. Electrospinning is the most current technology that is being used to form fibrous, porous material of varying but specified orientations. It would be interesting to use the characterization methodologies developed in this study to quantify and compare the porosity, pore size, and alignment of engineered muscle tissue obtained from electrospinning techniques to actual skeletal muscle.
5. REFERENCES


