

The Effects of Particle Size of Collagen and Mold Material on the Pore Structure of Freeze-Dried Collagen-GAG Scaffolds

by

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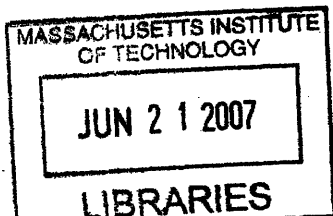
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Jenny K. Chan

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ABSTRACT

This study was performed to determine whether the particle size of the starting collagen powder or the material of molds used during freeze-drying had effects on the scaffold pore structure. Collagen particles were separated by size prior to slurry making using a sieve with 1000 μm openings, and scaffolds were made using both metal pans and polysulfone trays, two commonly used molds. The mean and variation of pore diameter and interconnectivity of freeze-dried scaffolds were compared to determine the relationship between particle size or mold material and the resulting pore diameter, for a specific same freeze-drying condition (*viz.*, temperature). Knowing these relationships will permit a better control of pore size during fabrication, allowing researchers to design scaffolds with greater predictability and specificity.

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Professor of Orthopaedic Surgery at Harvard Medical School

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1. Introduction

Porous sponge-like scaffolds play an important role in tissue engineering. Controlling scaffold pore diameter during production is essential for improving the scaffold's ability to aid in tissue regeneration. Collagen-glycosaminoglycan (GAG) scaffolds have been used for wound healing and tissue regeneration. They promote cell and tissue attachment, which depends partly on the pore structure of the scaffolds.

Pore sizes have been controlled through the cooling rate during the freezing step. This method, however, produces results with large error margins, which suggests other variables may also affect pore size. This project will explore two possible variables – size of collagen powder particles and materials of molds used during the fabrication of scaffolds. The results will build on existing techniques, such as constant cooling rates for pore homogeneity, to further improve control of pore structure.

2. Background

Collagen-GAG scaffolds have been applied to many areas of the body, such as nerves and skin, to induce tissue and organ regeneration. Because of this diverse application, scaffolds are often designed for their specific locations. Designing a scaffold involves controlling physical and structural properties, like the degradation rate and mean pore size.^{1,2} Restrictions placed on these parameters can be understood through the parameter's contribution to induced wound healing. This thesis project will focus specifically on the design of collagen scaffolds through the control of pore size.

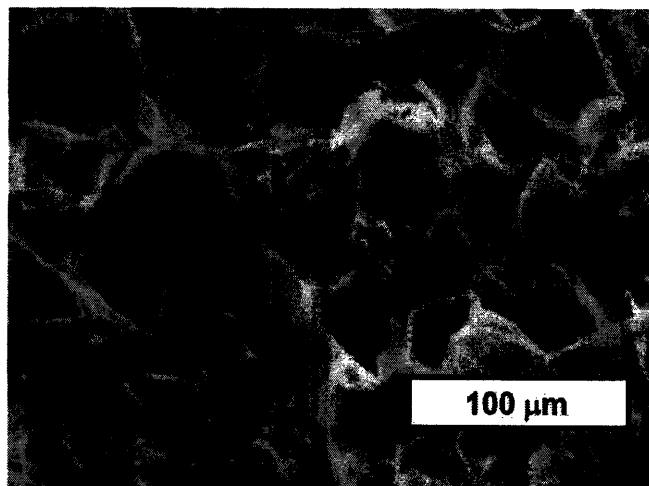


Figure 2.1 Scaffold used to promote skin regeneration⁷

2.1 Collagen-GAG Scaffolds in Wound Healing

Scaffolds (shown in Figure 1) are used to induce regeneration – the recovery of structure and function of tissues in organs.¹ Organs can be comprised of several tissue layers; for example, skin is made up of three tissue layers: epithelia, basement membrane and stroma. Certain individual tissues, such as the epithelial tissues, are capable of spontaneous regeneration following injury, even in adult mammals; other tissues are not.¹ By preventing fibroblasts from accumulating and aligning at the wound site, these scaffolds hinder contraction and the formation of scar tissue and also promote regeneration of the stroma. In addition, they provide a support structure analogous to the extracellular matrix to assist cell migration and adhesion, allowing for the formation of new tissue. This cellular activity depends on the scaffold's pore structure.

The average pore diameters of active scaffolds lie within a set range as a result of biological restrictions. The pores must be large enough for cells to migrate through yet small enough to retain a critical total surface area for sufficient cell binding.³ Cell attachment depends on the number of ligands, which is determined by two properties of the scaffold: the chemical composition and surface area. Both cell migration and attachment depend on the size of the scaffold's pores. According to Figure 2, substantial cell activity occurs at pore diameters of 20-125 μm ¹. Active scaffolds with pores in this range allow for regeneration to occur by increasing the wound's contraction half-life (slowing the rate of contraction). This range varies with chemical composition and cell type. Scaffolds with pores outside this range are inactive. Therefore, it is important to develop methods for controlling pore size during fabrication to produce active scaffolds.

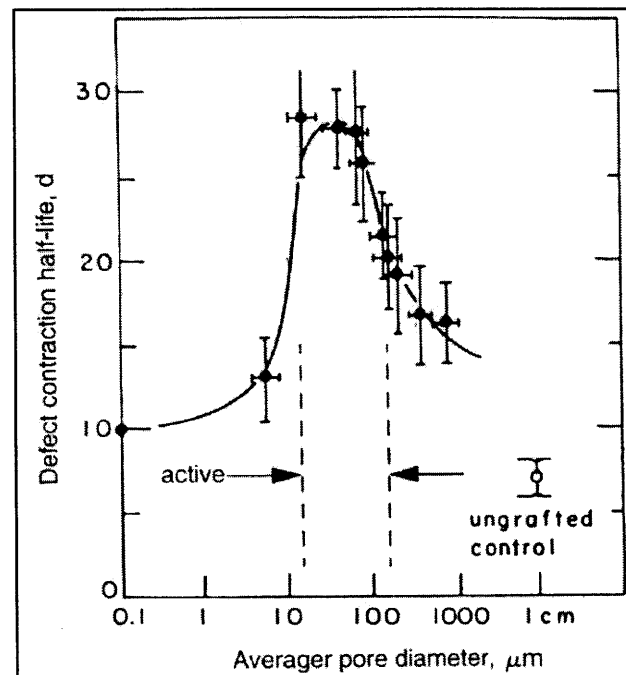


Figure 2.2 Pore sizes of active scaffolds (large contraction half-life – slow contraction rate) lie within a given range²

2.2 Current Method for Controlling Pore Size

Different cells bind preferentially to scaffolds with varying pore sizes. For example, endothelial cells prefer silicon nitride scaffolds with pores smaller than 80 μm while fibroblasts prefer pores larger than 90 μm .³ Therefore, the ability to produce scaffolds with specific pore sizes is essential for improving their efficacy.

There is currently only one way to control pore sizes when using the freeze-drying method. By lowering the final temperature, the formation rate of ice crystals increases and produces scaffolds with smaller mean pore sizes.^{3,4} This method, however, results in a large error of margins, which suggests other variables may affect the pore size. For example, when an average pore size of 109.5 μm is predicted, the scaffold produced has pore diameters deviating $\pm 18.3 \mu\text{m}$ from the average.

2.3 Objectives

This thesis explores the possibility of improving the control of pore size by regulating the collagen powder particle size used. Other pore characteristics will not be studied as vigorously, since pore shape and alignment have been greatly improved through modification of the freeze-drying method. By keeping the cooling rate constant during fabrication, more homogenous pore structures are formed.³ Uniform pore structures are preferable; studies suggest tissue synthesized in a scaffold with more uniform pore structures show superior biomechanical properties compared to those from a non-uniform pore structured scaffold.^{3,5} A second variable to be investigated is the material from which the mold is fabricated: polysulfone or metal. The supposition is that the difference in the thermal conductivity of the mold material would affect the slurry freezing process and thus the pore diameter.

3. Methods

This thesis involves the separation of collagen powder by size, fabrication of collagen-GAG scaffolds, and the preparation and application of pore analysis. Slurries composed of different types of collagen were blended, degassed, frozen, and sublimated to make scaffolds. Pieces of the scaffolds were removed for JB-4 embedding, sectioning, and staining for pore analysis preparation. The average pore size was then determined by conducting a pore analysis on the scaffolds.

3.1 Scaffold Fabrication

The process of fabricating scaffolds in this study involved three main steps. Collagen powder was first separated by size using a sieve. The collagen was then dissolved in 0.001N hydrochloric acid, blended and degassed to create slurry. Scaffold fabrication was completed by freezing the slurry and sublimating the ice crystals in a vacuum.

3.1.1 Separation of Powder by Size

Type I porcine collagen powder (Bio Gen, Geistlich Pharma AG, Wolhusen, Switzerland) was used for the analysis of particle size effect on scaffold pores. The powder was sifted in 4g portions using a sieve with openings of 1000 μ m, stainless cloth, brass frame, and 3-inch diameter (ASC Scientific). The powder was placed on top of the sieve, which was then fit into a 16oz. cup and covered with an upside-down cup lid that was taped to the cup as shown in Figure 3.1. The apparatus was then shaken at 260 rpm (Innova 2300 Platform shaker, New Brunswick Scientific) for 90 minutes in a 28 $^{\circ}$ C room. The particles above and below the sieve at the end of this process were stored in separate falcon tubes and placed in the desiccator.



Figure 3.1 1000 μ m sieve and shaking apparatus used

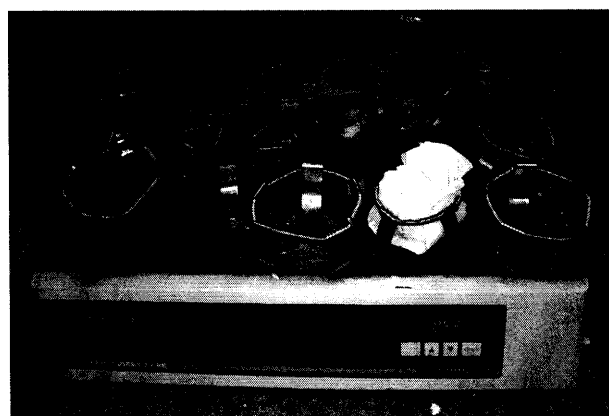


Figure 3.2 Shaking platform used (sifting apparatus in top right corner)

3.1.2 Making Slurry

Slurry was made from the type I porcine collagen powder as given, the two divisions of collagen resulting from sifting, and the cut collagen sheets. The protocol used in this experiment referenced the slurry making procedure described by O'Brien et al.³

A magnetic spinner was used to dissolve the collagen in a beaker of 0.001N hydrochloric acid to create a 1% concentration of slurry. Small amounts of 6N hydrochloric acid were added to the solution to reduce the pH level to 3. The solution was then mixed at 14,000 rpm for two hours using an overhead blender (IKA Ultra Turrax T18). The blending was interrupted after 1hr to remove the slurry and reduce the pH level to 3. The slurry was maintained at 4°C in a cylindrical metal container using a refrigerated bath circulator (Neslab RTE-100) to prevent denaturation of collagen fibers during the blending (Figure 3.3)



Figure 3.3 Blending apparatus with cooling system attached

When the slurry was finished mixing, it was transferred into falcon tubes and placed in a centrifuge. The tubes were spun at 2500 rpm for 15 min at 4°C in 25 mL quantities to remove the bubbles created during the blending. After centrifugation, the surface bubbles were removed using a metal spatula and the collagen pellet was resuspended. The solution was mixed with a pipette until it became homogenous. The slurry was stored at 4°C or used immediately to make scaffolds.

3.1.3 Freeze-Drying

After the slurry preparation was complete, it was transferred into a 5 in. x 5 in. stainless steel pan or a 5.75 in. x 1.5 in. polysulfone tray using a pipette. Any visible bubbles were removed using a metal spatula. The pans were then subjected to the modified freeze-drying method developed by O'Brien F.J., et al. This method incorporated a constant cooling rate during the freezing step to produce more homogenous pore structures.³

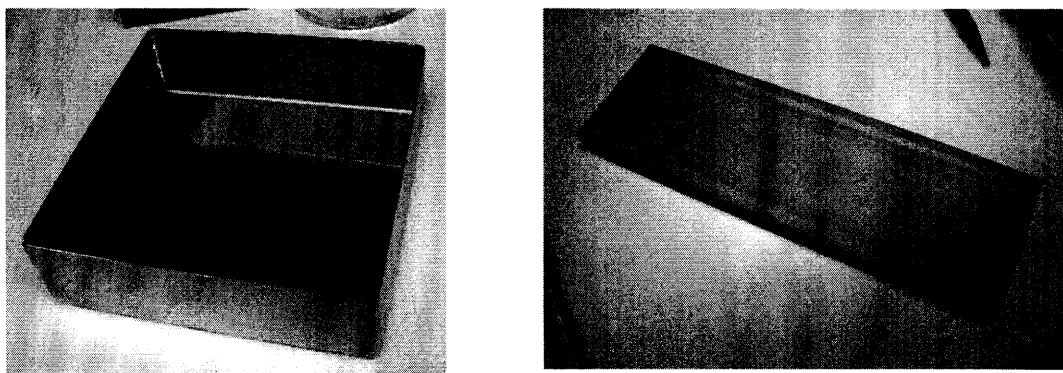


Figure 3.4 Stainless steel pan and polysulfone tray used for freeze-drying process

The shelf temperature in the freezer dryer (AdVantage Benchtop Freeze Dryer with Wizard 2.0 control system, VirTis, Labrepco, Gardiner, NY 12525) was reduced to 4°C before the pan containing slurry were placed inside. The freeze-drying program selected for this study began with a temperature hold at 4°C for 30min to bring the slurry to a uniform temperature. The freezing process was then initiated by ramping the shelf temperature from 4°C to -20°C over a 180 min time frame. The temperature was held at -20°C for 60min to complete the freezing step.

The program then proceeded to the drying step by turning on the vacuum. When the vacuum pressure reached 200 mTorr, the chamber temperature was raised to 0°C and maintained for 17 hours. A secondary drying step followed by increasing the temperature to 20°C and holding for 30 min. These conditions induced sublimation of ice crystals in the slurry, resulting in pore formation.

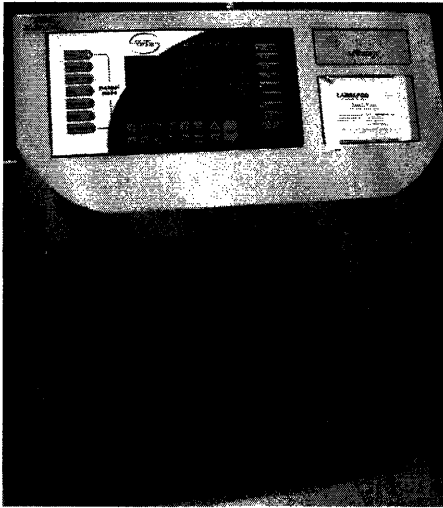


Figure 3.5 VirTis freeze dryer

The freeze-drying process was completed when the vacuum was released. The scaffold was removed from the pan and stored in an aluminum foil envelope that was placed into a desiccator at room temperature.

3.2 Pore Analysis

Once scaffolds were created, they could be prepared for pore size analysis. Specimens were cut from the scaffolds sheets using a biopsy punch and then infiltrated and embedded in a JB-4 solution. The embedded samples were then sectioned using a microtome, placed on microscope slides, and dyed with aniline blue. Images of sectioned scaffolds were captured on an optical microscope using a magnification of 4X. These images were processed and average pore diameter calculated using a computer program. The entire analysis process took approximately one week.

3.2.1 Cutting Scaffold Specimens

Sections were removed from the collagen scaffolds for pore analysis. Specimens were cut under a sterile hood using biopsy punches with 8mm diameters (Miltex). Since the surrounding scaffold area tended to tear when the cutting edge became dull, a new biopsy punch was used after every 4 to 5 punches.

Nine samples were taken from scaffolds made using the metal pan. The scaffold was cut at the four corners about 1 in. from each edge, once in the center, and four times around the center. Seven samples were taken from the scaffolds made using the polysulfone trays. All samples were centered along the width with an approximate spacing of 1 cm along the length. Scaffolds were cut once in the middle and three times on both the right and left halves. All cuts were made as outlined or in the most homogenous surrounding regions.

All specimens were transferred to falcon tubes using tweezers. Metal pan scaffold specimens were contained in 50mL tubes while those from polysulfone trays were put in 15mL tubes. Tubes were then stored in a desiccator at room temperature or equilibrated for the next step.

3.2.2 Specimen Embedding

A series of steps were performed on scaffold specimen to prepare them for pore analysis. The discs were first equilibrated and infiltrated to increase their stiffness, then embedded in a JB-4 solution for the microtome sectioning. These slices were then stained for collagen using aniline blue, making the pores easily distinguishable. All solution mixing was performed under a hood at room temperature.

Samples were equilibrated for 12 hours at 4°C in a solution containing equal amounts of 100% ethanol and catalyzed JB-4 solution A. The catalyzed JB-4 solution A was made by dissolving 0.3125g of JB-4 A catalyst (Benzoyl Peroxide, polysulfoneized, Cat. No. 02618-12, Polysciences) in 25mL of JB-4 A monomer solution (Cat. No. 0226A-800, Polysciences). The falcon tubes were placed on a rotator during equilibration.

Specimens were then infiltrated using catalyzed JB-4 solution A. The solution was changed every 12 hours until infiltration was completed after 3 to 4 days. Tubes were placed on a rotator and kept at 4°C during infiltration. At the end of this process, samples were translucent and sunk to the container's bottom.

Infiltrated scaffolds were then removed from the tubes and embedded in a JB-4 solution. Samples were cut across a chord, creating a piece approximately 2/3 the size of the original disc. This slice was placed in the rectangular opening of a plastic embedding mold with the circular area parallel to the tray's bottom (figure 3.6). The rectangular region was then filled with an embedding solution containing JB-4 catalyzed solution A and JB-4 solution B (Cat. No. 0226B-30, Polysciences) at a ratio of 25:1. Plastic block holders (EBH-2) were placed face down over the samples and embedding solution was added to the center hole until liquid was visible around the holder's outer circumference. Tweezers were used to push floating scaffolds to the bottom until solution became viscous after 30-45 minutes. The tray was kept at 4°C overnight to allow the solution to harden. The embedding blocks (Figure 3.6) were popped and allowed to dry for at least 12 hours before sectioning.

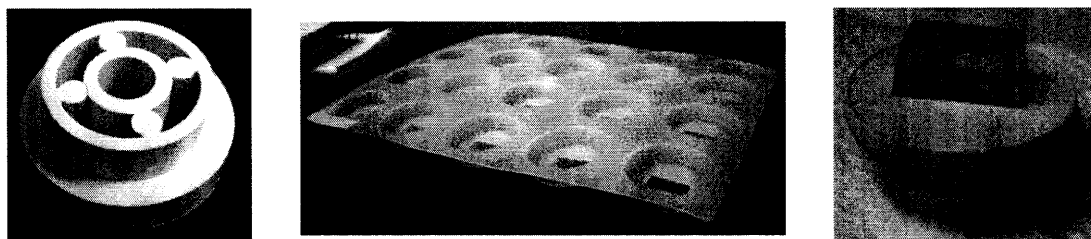


Figure 3.6 Plastic block holders are placed in the tray containing scaffold slices and filled with embedding solution to create a block for sectioning.

3.2.3 Specimen Sectioning and Staining

The blocks of embedded specimen were sectioned in slices of 10 μ m thickness using a microtome (Finesse, Figure 3.7). Two sections of each specimen were made since the slides were sometimes soiled during application of coverslip adhesive. Each section was placed in a water bath containing a few drops of ammonium hydroxide before being set on glass microscope slides. The slides were dried overnight at room temperature.

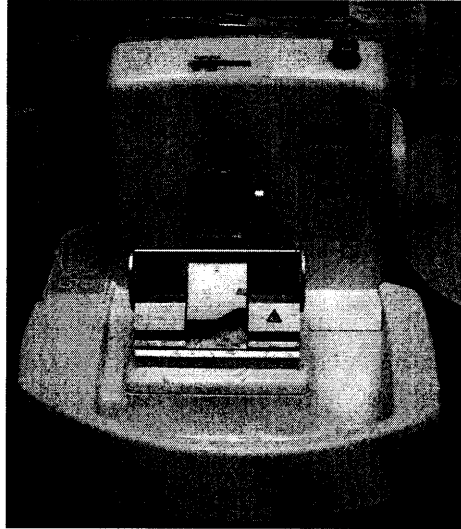


Figure 3.7 Microtome used for sectioning

The sections were then dyed using 2.5% aniline blue (Fisher Cat. # A-967), which turned the collagen fibers blue. Less than 100mL of aniline blue was filtered to remove any unwanted particles from previous staining. The slides were dipped into the aniline blue solution for 2 minutes to stain the collagen. Background staining was removed by dipping the slides into 1% acetic acid solution (Fisher Cat. # A A507-500) for 1 minute, 95% alcohol 5 to 10 times, and 100% alcohol 5 to 10 times (Figure 3.8). The slides were immediately mounted using Cytoseal 60 (Electron Microscopy Sciences Cat. # 18006) and microscope coverslips.

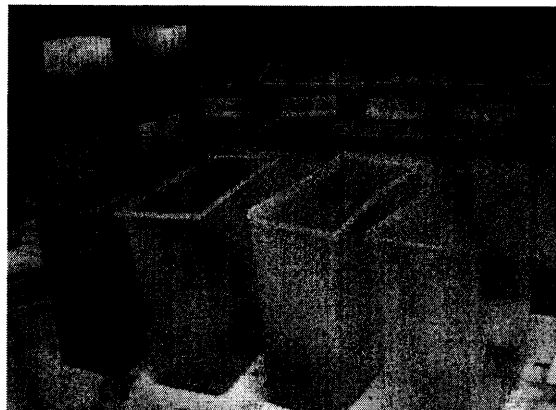


Figure 3.8 The plastic microscope slide holder used to stain samples simultaneously in the different solutions

3.2.4 Determining Pore Diameters of Scaffolds

The analysis used to determine average pore diameters follows a standard scaffold pore size analysis protocol for image acquisition and linear intercept analysis.^{2,3} The stained slides were viewed with an optical microscope (Nikon Optiphot, Japan) under 4X magnification. Images of the sectioned collagen were captured with a CCD color video camera (Optronics Engineering, Inc., Goleta, GA) and processed using a Scion Image analysis software. The microscope scale was calibrated using an image of a standard 1mm band to determine the relationship between pixel size and metric length. A program was applied to the sectioned scaffold image to calculate the major and minor axes of the best fit-ellipse from the average distance between struts along each line (collagen fiber). The axes lengths were then used to determine the average pore size of the section.

4. Data Analysis and Results

The study examines the possible effect of collagen powder size and mold material on the average scaffold pore diameter. Five scaffolds were produced from three different slurries made using the porcine collagen powder as given (control), large powder particles, and the small powder particles. Three scaffolds, one per different slurry type, were made using the metal pan. Two scaffolds, one from the large particle powder and one from the small particle powder, were made using the polysulfone trays. Six specimens from each scaffold produced were sectioned for pore analysis to determine the average pore diameter for the scaffold. Pore sizes were then statistically compared to each other using StatView to determine if the studied variables had a significant effect on pore size.

4.1 Sifting Powder

Collagen powder was first separated by size using a 1000 μ m sieve. Particles were considered "large" if they did not pass through the sieve and "small" if they were found below the sieve after the sifting process. An initial collagen powder sample of 4g produced approximately 1.8g of large particles and 1.9 g of small particles after 90 minutes of shaking at 260rpm. Sieves with openings of 106 μ m and 20 μ m were also tested. No powder was able to pass through either sieve at high speeds or over long periods of shaking.

The large powder had a flaky texture that allowed some large particles to be easily seen while the small powder was very fine and static (Figure 4.1). Although the texture differences between the two size groups were determined by eye, the small and large collagen particles were difficult to distinguish using an optical microscope. The tendency for collagen fibers to clump made the exact size of the particles difficult to quantify.

Slurry made using the large particle powder was observed to have a higher viscosity than slurry made using small particle powder. The effect of slurry viscosity on scaffold pore size not known, however, the centrifugation process should be adjusted for the appropriate viscosity to ensure all bubbles have been removed.

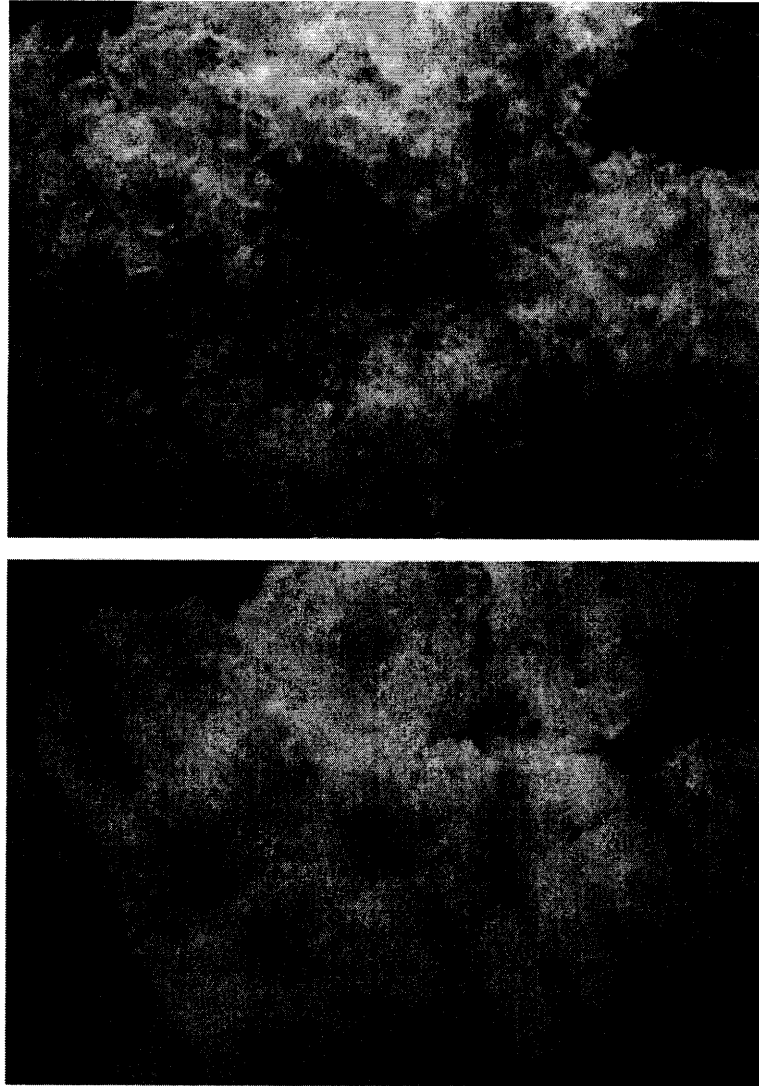


Figure 4.1 Large collagen powder (above) remained above the sieve while smaller powder (below) passed through the 1000µm openings

4.2 Effect of Collagen Particle Size

The specimens from all five scaffolds were combined to study the overall effect of particle size on pore diameter regardless of mold material used. The average pore diameters of the combined scaffolds, as shown in Figure 4.2, were $193 \pm 72 \mu\text{m}$ (mean \pm standard deviation), $167 \pm 64 \mu\text{m}$, and $126 \pm 29 \mu\text{m}$ for the large particle, small particle, and control scaffolds respectively. One-factor analysis of variance (ANOVA) indicated that there was no significant effect of particle size on the pore diameter (p-value of 0.122). The coefficients of variation were relatively high (38% for large and small particle scaffolds), suggesting that there was some inconsistency in freeze-drying process in each group of scaffolds. Our results are inconclusive because of the large uncertainty values.

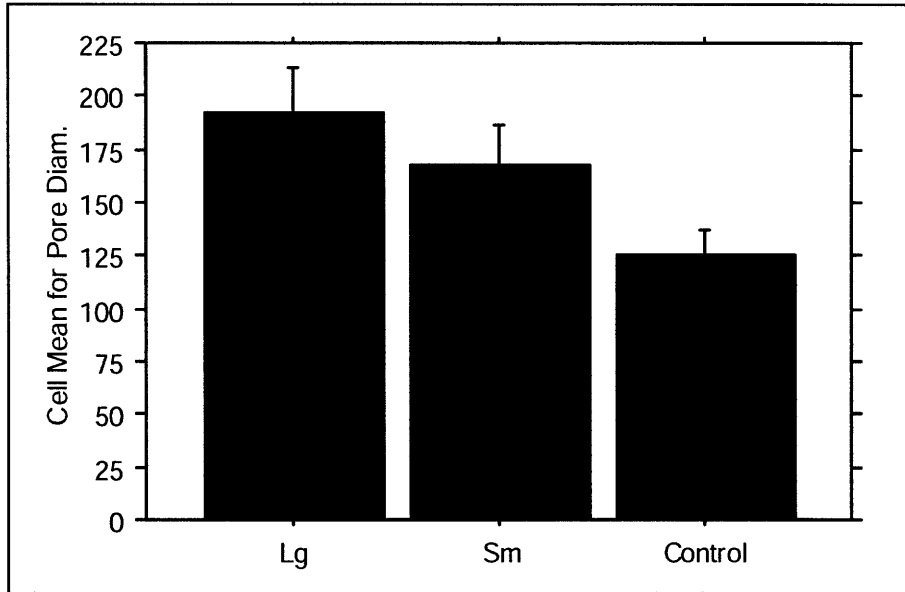


Figure 4.2 Average pore diameters (μm) of combined scaffolds for different collagen particle sizes. Error bars indicate one standard deviation.

Scaffolds made using the metal pans contained similar pore sizes regardless of starting collagen particle size. Large particle and control scaffolds had nearly identical pore diameters of $127 \pm 29 \mu\text{m}$ and $126 \pm 29 \mu\text{m}$ respectively, while the small particle scaffold diameters were slightly smaller at $114 \pm 37 \mu\text{m}$ (Figure 4.3). The one-factor ANOVA p-value of 0.75 was relatively high and the power value of 0.087 was very low. These values indicated the probability of collagen particle size having an effect on pore size was very low when scaffolds were made using metal pans.

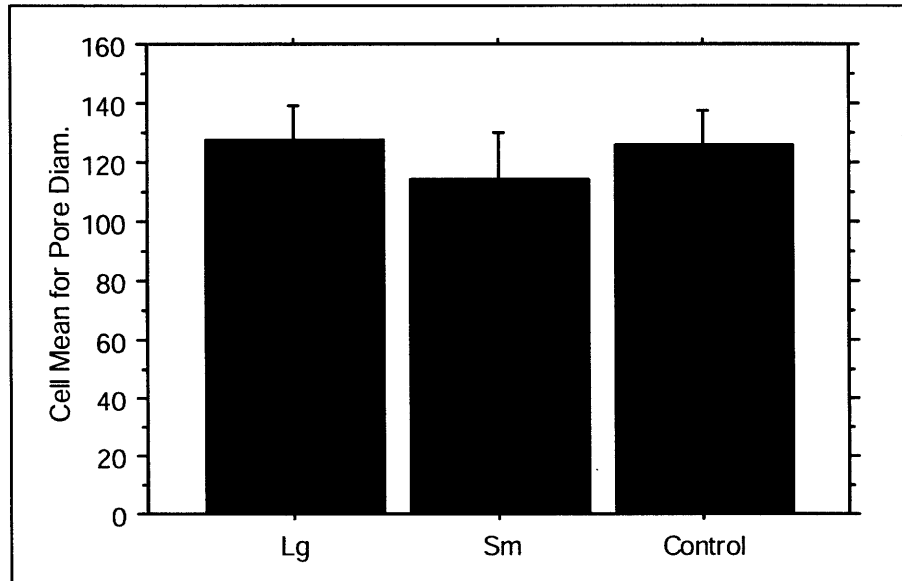


Figure 4.3 Average pore diameters (μm) of metal pan scaffolds for different collagen particle sizes. Error bars indicate one standard deviation.

Scaffolds made using the polysulfone trays, however, showed a difference between the large and small particle sizes. Large particle scaffolds had a larger average pore diameter of $258 \pm 22 \mu\text{m}$ than the small particle scaffolds with $221 \pm 30 \mu\text{m}$ diameters as shown in Figure 4.4. This difference in pore sizes has a p-value of 0.0325 (confidence level greater than 95%), making it statistically significant.

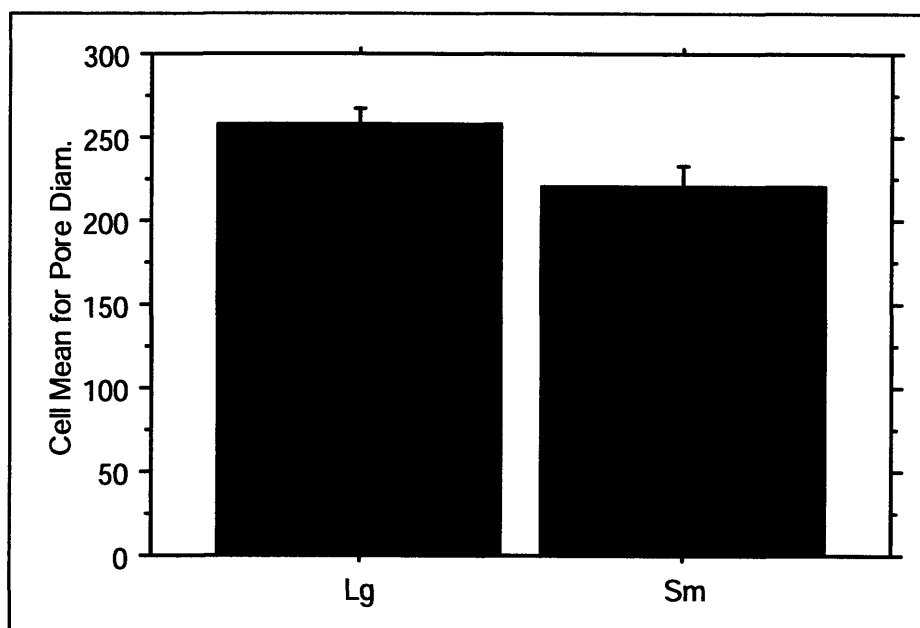


Figure 4.4 Average pore diameters (μm) of polysulfone tray scaffolds for different collagen particle sizes. Error bars indicate one standard deviation.

Although these results indicate larger starting collagen particles produce scaffolds with larger pore sizes, the power value of 0.608 suggest some uncertainty in this conclusion. The probability that the null hypothesis is false, that there is a relationship between collagen particle and scaffold pore size, is lower than the ideal value of 0.8. To increase this probability, more than 6 specimen should be taken from each scaffold. A power calculation using the mean difference ($37 \mu\text{m}$) and standard deviation ($30 \mu\text{m}$) from the data above indicated that a sample size of 9 was required to obtain a power value greater than 0.8.⁶

4.3 Effect of Mold Material

The specimens from all four large and small particle scaffolds were combined to study the overall effect of mold material on pore diameter, without considering the initial powder size. Polysulfone tray scaffolds were not made using the control solution, so the metal tray control scaffold was not included in the mold material analysis.

The average pore diameters of the combined scaffolds, as shown in Figure 4.5, were $121 \pm 32 \mu\text{m}$ and $239 \pm 31 \mu\text{m}$ for the metal pan and polysulfone tray scaffolds respectively. The polysulfone tray scaffolds had a statistically significant larger pore size than the metal pan scaffolds. The Student's t-test produced a p-value less than 0.0001. The high confidence level was complemented by a high power level of 1. This suggested that the null hypothesis was false, that the mold material had an effect on the pore size of scaffolds. In addition, the coefficient of variance did not exceed 26% (data collection was consistent) and the standard error was less than 10%. All statistical values indicated that using polysulfone tray scaffolds resulted in scaffolds with larger pore diameter.

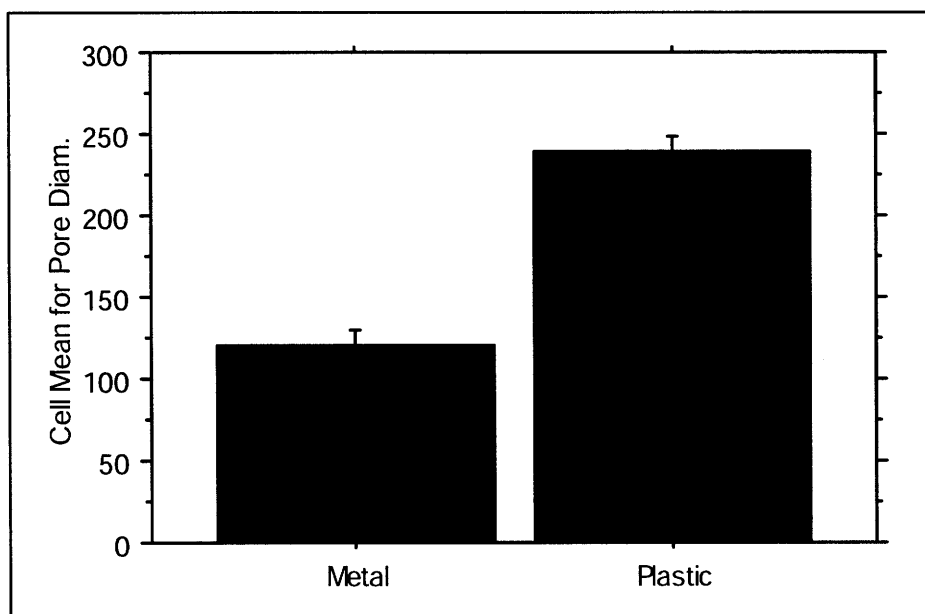


Figure 4.5 Average pore diameters (μm) of combined collagen particle sizes for metal and polysulfone molds. Error bars indicate one standard deviation.

There was a large difference between the scaffolds made using the metal pans and ones made using the polysulfone trays. This difference was seen in both large and small collagen particle scaffolds, as seen in Figure 4.6. In scaffolds made from large particle collagen, average pore diameters were $127 \pm 29 \mu\text{m}$ and $258 \pm 22 \mu\text{m}$ when metal pans and polysulfone trays were used respectively. The p-value for these results was less than 0.0001 and the power value was 1.0.

The effects of different mold materials was slightly less in small particle scaffolds, however, the differences were still very significant statistically. The average pore diameters were $114 \pm 37 \mu\text{m}$ and $221 \pm 12 \mu\text{m}$ for metal pans and polysulfone tray scaffolds respectively. These values had a very high confidence level as the p-value was 0.0003 and the power value was 0.999.

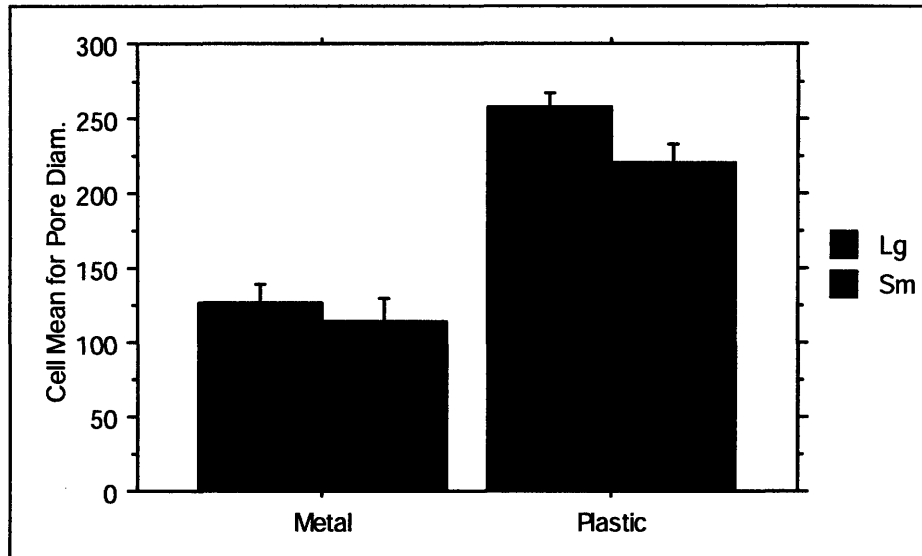


Figure 4.6 Average pore diameters (μm) of large and small collagen particle sizes for metal and polysulfone molds. Error bars indicate one standard deviation.

5. Conclusion

This study was conducted to determine whether size of the starting collagen powder or material of scaffold molds used for freeze-drying affected the size of scaffold pore diameters. Collagen powder was successfully separated into two size groups using a sieve with $1000\mu\text{m}$ openings. Particle size had minimal effect, if any, on scaffolds made using metal pans. Scaffolds made using polysulfone trays showed a difference in average pore size based on collagen particle size. Larger particles produced pores with greater diameters than the pores produced by the smaller particles; however, the difference was not statistically significant. A larger sample size is required to increase the power value, the probability that the results refute the null hypothesis.

The material of freeze-dryer molds had a significant effect on the scaffold pore sizes regardless of starting particle size. Scaffolds made using the polysulfone trays had a larger mean pore size than those made using the metal trays. Although the results were statistically acceptable, further studies should be conducted before any conclusions are made. Average pore diameters of the polysulfone mold scaffold were unusually large, almost twice the length of expected pore diameters.

One possible source of error stems from the temperature variability of the freeze-dryer shelf during freezing and sublimation. Switching the positions of the metal and polysulfone molds in the freeze-dryer during future experiments may provide more information regarding this issue. Also, specimen from the polysulfone tray scaffolds were infiltrated using 15mL falcon tubes instead of 50mL tubes. The narrow girth of the 15mL tubes made removal of old infiltration solution difficult and scaffolds may have

been flattened during the process. This possibility can be avoided using 50mL tubes during infiltration.

Despite these possible sources of error, the results do suggest a relationship between the variables studied and the scaffold pore size. Further study of these relationships would determine if collagen particle size and mold material are variables that need to be controlled during scaffold fabrication. This would potentially allow researchers to design scaffolds with greater precision by accounting for such variables.

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