THERMO-MECHANICAL INTERACTION WITHIN ENGINEERED TISSUE DURING FREEZING

by

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ABSTRACT

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Cryopreservation is the preserving of biological tissues at sub zero temperature. There are many useful applications to cryopreservation such as storing donated organs till they are needed rather than letting them go to waste. When tissues are frozen and thawed, their original functionality and vitality are not usually maintained. It's been shown that when tissue is frozen its mechanical properties change, such as tensile strength and modulus. Each tissue has an extra cellular matrix made up of many collagen fibrils that is responsible for the structural integrity of the tissue. This study is to investigate the structural changes of ECM by freeze/thaw by use of scanning electron microscopy and thermo-mechanical analysis of tissue deformation during freezing. In this experimental study, three types of engineered tissue containing human fibroblast cells were examined for microstructural changes in the collagen ECM. The three types of engineered tissue had variable collagen and cell density. These densities were 1) 3mg/ml of collagen and 200,000 cells/ml 2) 3mg/ml of collagen and 400,000 cells/ml, and 3) 6mg/ml of collagen and 200,000 cells/ml. Changes in fibril area, mean void area and fibril diameter were to be examined. In this study a thermo-mechanical finite element model was developed to predict the location of the freezing front and possible the deformation rate and dilatation of the engineered tissue during freezing. The results indicated that freezing of ET causes the increase of mean void area of voids between fibrils, and the decrease in the average fibril area and the

diameters of the fibrils increased. For the model it was found that the location of the freezing front could be predicted, but dilatation and deformation rates could only be predicted close to the order of magnitude of the experimental results.

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CHAPTER 1

INTRODUCTION

1.1 Cryopreservation

The idea of freezing tissues to preserve them has been around for a long time. The idea is that by freezing tissues to a point where metabolic processes are almost stopped, decay is almost stopped altogether. The tissue can then at a later time be thawed and used for transplantation/implantation surgeries. The practical purposes include preservation of donated organs for months till needed, and preservation of ovarian tissue for young women going through chemotherapy until their treatments are over. Providing this preservation services would save lives and give people hope for natural births where hope would otherwise have been lost.

1.1.1 Current Tissue Preservation Techniques

Current methods of tissue cryopreservation include vitrification and slow freezing. These methods show great promise and have had some success in transplanting tissue and cryopreservation of ovarian tissue and oocytes. [1]

1.1.1.1 Vitrification

The vitrification method of cryopreservation is the ultra-rapid freezing (15,000C-30,000C/min) of tissue with high concentrations of cryoprotectants. This method prevents the formation of ice crystals, but increases the toxicity the tissue must encounter during the loading and unloading of the cryoprotectants. The benefit to this method is that intracellular and extracellular ice crystallization is avoided as the water turns into its glassy state with no crystallization. This method requires rapid cooling and high concentration of cryoprotective agents which can be difficult to load in thick tissues. The cryoprotective agents lower the

freezing temperature assisting the circumnavigation of intercellular and interstitial fluid crystallization.

1.1.1.2 Slow Freezing

Another method currently being used is that of slow freezing. Slow freezing is the method of cooling tissue at a set rate after tissue has been loaded with cryoprotectants. The cryoprotectants lower the freezing temperature of the interstitial fluid between cells and in cells. As the cells are loaded with cryoprotectant the water leaves the cells, dehydrating them, meaning less ice formation inside the cells. The interstitial fluid freezes at a lower temperature as well as causing less ice formation. The cooling rate and concentration of cryoprotectant must be balanced or damage by ice formation or toxicity will begin to kill large parts of the tissue. Still, much less cryoprotectant is needed in slow cooling than in vitrification. The cooling and loading rates are tissue dependant for slow freezing [2].

1.2 Previous Theoretical and Experimental Studies

A recent study of the mechanical property of arteries gave rise to interesting questions [3]. Arteries were tested for modulus, length, diameter and weight. The arteries measured were frozen with no protection, and frozen with cryoprotectants. Freezing caused increased diameter of the arteries, drop in weight and a small change in the linear modulus. Cryopreservated samples had similar diameters but decrease in linear modulus. The effects of freezing on human tissue were observed but the mechanics are unknown. What was observed was that the nuclei of the cell were condensed during freezing indicating injury to the cells. Other studies [4] have shown that changes to mechanical properties such as maximum stiffness and ultimate tensile strength of tissue are dependent on cooling rates. Using cooling rates of 0.5, 5, 20, 40, >1000 C/min when compared to control samples of type I collagen with fibroblast cells shows that maximum stiffness can be higher or lower than the control, depending on the cooling rates used to freeze the ET. In this same study it was shown that end temperatures of preservation also had an effect on the mechanical properties of tissue. Specifically that tissue frozen to -80 C

showed after 72 hours of thawing a significant increase in modulus and ultimate tensile stress which suggest a cell-mediated recovery mechanism. Tissue frozen to -160 showed adverse affects to the same mechanical properties when thawed after 72 hours.

1.3 Introduction to WetSEM capsule technology

The use of WetSEM, or the Quantomix Capsules, technology is desired so that biological tissue samples can be viewed in a fully hydrated state using SEM. A fairly new technology, it's not widely used for research but has found its way into publication [7] [8]. The idea is simple in that cells or tissues are placed on a thin membrane that allows the passage of electrons. The membrane acts as a window into an air tight capsule. Low contrast materials can be imaged, but in order to make them visible for analysis staining agents such as uranyl acetate or osmium tetra-oxide can be used to obtain higher resolution and higher contrast [9]. The advantages of these capsules is that the engineered tissue (ET) used in this study went through fewer sample preparation processes than that of previous studies. [6] The samples do not have to be coated in a conductive material like other biological samples in traditional SEM imaging. In SEM the sample chamber is evacuated of all air, creating a vacuum. Normally a wet sample would have to be dehydrated then dried in order to be unaffected by the vacuum in a SEM chamber. The capsule creates an air tight chamber that prevents the escape of air or liquid allowing the sample to be close to atmospheric pressure, and its original hydration.

1.4 Current Challenges

The desired outcome of cryopreservation is that tissues can be stored then transplanted and retain their ability to perform their original function. The preservation process must not ruin the functionality or viability of the tissues that are preserved. One observed problem to functionality is the toxicity brought on by cryoprotectant loading and unloading. This toxicity damages cells, making the tissue unfit for transplantation. This is especially difficult to deal with in thick tissues [10]. Some of the challenges to functionality include the changes in mechanical properties due to the freeze thaw process as stated above. One of the large unknowns is the

cause of the change in mechanical properties brought about on tissues by the freeze/thaw (F/T) process. Though the changes in mechanical properties have been observed, a better understanding of the causes of these changes is needed. Future studies would hopefully be able to take this knowledge and update the current protocols to mitigate the causes of these changes.

1.5 Objective of This Study

Though the cells of tissue may survive and repair themselves over time, it's possible that the tissue still isn't functional. The objective of this study is to quantify changes to the extracellular matrix due to freezing and thawing. The extracellular matrix (ECM) is a mechanical scaffold of biological tissues to which the functional properties of a tissue are dependent upon. To know what is happening to the ECM microstructure during the F/T process is an important step in understanding the cause of the mechanical changes in tissue before and after freezing [11]. The ECM itself is composed of a number of collagen fibrils. Knowing this, one way to quantify changes to the ECM would be to compare the collagen fibril's condition before and after freezing. The criteria to be observed is the amount of collagen material present in a given area. Also to be quantified is the number of fibrils in an area and also the change in diameter of fibrils before and after freezing. This work has been done before in previous studies [6], so to bring the work to its next step; the ECM will be populated with human fibroblast cells. Fibroblast cells are common throughout most tissue and, in these ETs, grab hold, compact, and strengthen the ECM. Fibroblast cells also synthesize collagen and are important to the structural integrity of the ECM.

CHAPTER 2

THEORETICAL BACKGROUND

2.1 Problem Statement w/ Schematic of Computational Geometry

Part of this study is to model the mechanical effects of the freezing process in the ET. What is desired to be observed is the strain rate brought on by the density change of water to ice. The ET to be made for these experiments has a thin rectangular geometry. ET resembles a gel like material in macroscopic appearance, but is actually a porous network of collagen fibrils held together by human fibroblast cells. Interstitially the structure of collagen and cells is filled with an isotonic saline and small amount of culture medium for the fibroblast cells. To model the freezing process of this engineered tissue, the mechanical properties of the collagen and the thermal properties of the collagen and interstitial fluid were obtain from other studies [11-14] and used to estimate the properties of the engineered tissue. These will be described in greater detail later on. First for modeling, the geometry of the ET is shown below.

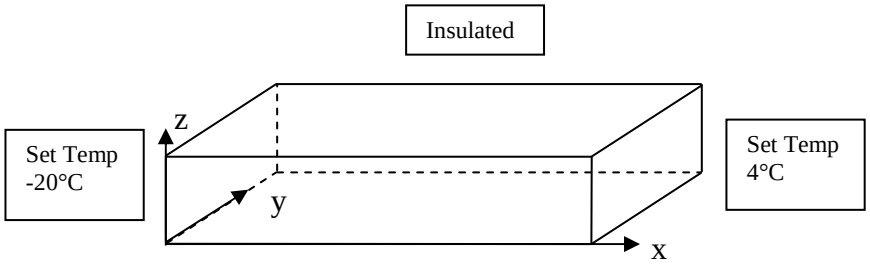


Figure 2.1 The thermal boundary conditions for the engineered tissue model. The dimensions of the engineered tissue are 50 mm x 20 mm x 2 mm in the x, y and z directions respectively.

2.1.1 Thermal Boundary Conditions and Initial Conditions

The ET is frozen due to a temperature gradient across -20℃ and 4℃ which gives us the two thermal boundary conditions of the ET. It was assumed that conduction would be the major form of heat transfer so that convection of the surrounding air would not be included in

this model. The model was also split in half parallel to the x-axis with the assumption that both halves are symmetrical. The initial conditions of the model are set so that thermal expansion due to temperature is zero.

2.1.2 Mechanical Boundary Conditions

The mechanical boundary conditions were a bit constrictive. Naturally the surface parallel to the x-plane at x=0 was fixed in the x direction but free in the y and z directions. Free in this case meaning allowing displacement in those directions and fixed meaning no displacement was allowed. The long surface parallel to the y plane at y=0 was fixed in the y-direction, but not in the x or z directions. This particular surface represents the line of symmetry. The bottom surface was fixed in all directions. It's known that some friction exists between the ET and the surface of the glass slide it sits on, but rather than find it experimentally it was thought best to assume there was no movement.

2.2 Governing Equations

The governing equations for transient thermal problems rely on the energy equation. Since the ET is approximated as a composite material with a solid and liquid phase consisting of collagen and interstitial fluid respectively which is mostly saline, the equations must account for the material properties of both. It must also be taken into account that all material properties are temperature dependant. The feasibility of modeling tiny fibrils in a volume of saline solution dictated that modeling the solid and liquid phase together as a homogenous composite material would be a great simplification to the modeling of this problem. The governing equations are separated into thermal and mechanical equations. Ansys uses the standard energy equation as below.

$$\rho(T)\,c(T)\frac{\partial}{\partial t}[T] = L^{T}[K(T)T] \qquad \text{Eq. (2.1)}$$

L in this case is a vector operator to take into account the 3-d heat flow seen in equation 2.2. This standard energy equation allows for the input of the constant temperature boundary conditions.

$$L = \begin{bmatrix} \frac{\partial}{\partial x} \\ \frac{\partial}{\partial y} \\ \frac{\partial}{\partial z} \end{bmatrix}$$ Eq. (2.2)

In order to account for the phase change of the material Ansys correlates the specific heat of the energy equation to an inputted enthalpy table. This allows for the phase change of the interstitial fluid to be accounted for without changing the energy equation to a two phase pair of equations or using the enthalpy equation formulation [5].

To calculate stress and strain Ansys separately calculates the thermal and mechanical strain to get the total strain. The stress is related to the strains by:

$$\{\sigma\} = [D]\{\varepsilon^{el}\}$$ Eq. (2.3)

where:

$\{\sigma\}$ = stress vector = $\left[\sigma_x\ \sigma_y\ \sigma_z\ \sigma_{xy}\ \sigma_{yz}\ \sigma_{xz}\right]^T$

[D] = elasticity or elastic stiffness matrix or stress-strain matrix which is assumed to be symmetric

$\{\varepsilon^{el}\} = \{\varepsilon\} - \{\varepsilon^{th}\}$ = elastic strain vector (output as EPEL)

$\{\varepsilon\}$ = total strain vector = $\left[\varepsilon_x\ \varepsilon_y\ \varepsilon_z\ \varepsilon_{xy}\ \varepsilon_{yz}\ \varepsilon_{xz}\right]^T$

$\{\varepsilon^{th}\}$ = thermal strain vector

Equation 1 is inverted to give the following relations ship between the total strain and stress vector.

$$\{\varepsilon\} = \{\varepsilon^{th}\} + [D]^{-1}\{\sigma\}$$ Eq. (2.4)

For the 3-D case, the thermal strain vector is

$$\{\varepsilon^{th}\} = \Delta T \begin{bmatrix} \alpha_x^{se} & \alpha_y^{se} & \alpha_z^{se} & 0 & 0 & 0 \end{bmatrix}^T$$ Eq. (2.5)

where:

α_x^{se} = secant coefficient of thermal expansion in the x direction
$\Delta T = T - T_{ref}$
T = current temperature at the point in question
T_{ref} = reference) temperature

The flexibility or compliance matrix, $[D]^{-1}$ is:

$$[D]^{-1} = \begin{bmatrix} 1/E_x & -\nu_{xy}/E_x & -\nu_{xz}/E_x & 0 & 0 & 0 \\ -\nu_{yx}/E_y & 1/E_y & -\nu_{yz}/E_y & 0 & 0 & 0 \\ -\nu_{zx}/E_z & -\nu_{zy}/E_z & 1/E_z & 0 & 0 & 0 \\ 0 & 0 & 0 & 1/G_{xy} & 0 & 0 \\ 0 & 0 & 0 & 0 & 1/G_{yz} & 0 \\ 0 & 0 & 0 & 0 & 0 & 1/G_{xz} \end{bmatrix} \quad \text{Eq.(2.6)}$$

where typical terms are
E_x = Young's modulus in the x direction
ν_{xy} = major Poisson's ratio
ν_{yx} = minor Poisson's ratio
G_{xy} = shear modulus in the xy plane

Ansys discretizes equations thermal and mechanical equations and boundary conditions to be solved in finite element form. Using the solution to the FE model Ansys will output the desired results of deformation rate, strain rate and dilatation. To calculate the deformation rate and strain rate at a given time step, the strain or deformation for the time step before and after are subtracted from each other and divided by two times the time step. The time step used in these calculations is 2 seconds. To obtain the dilatation, the strain rate in the x, y, and z directions were all added together. Dilatation itself is a rate at which the volume changes.

2.3 Solution Procedure Using Ansys

Ansys is a powerful tool used to solve mechanical and thermal effects of freezing on the ET. In figure 2.2 below a flow chart gives the major steps in Ansys script to get the solution over time for the mechanical and thermal effects. The key final calculations taken from Ansys are strain, deformation, and the temperature profile.

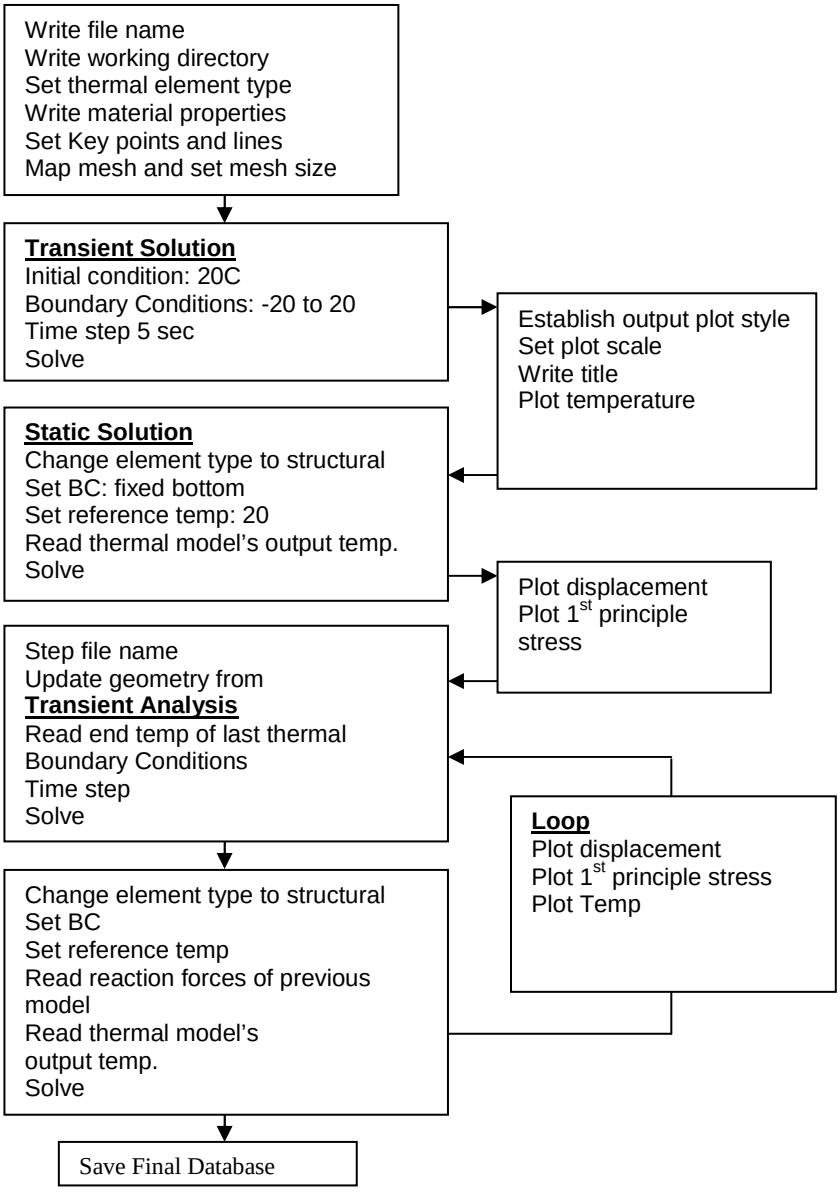


Figure 2.2 Flow chart representing imputes and calculation process for Ansys calculation.

The ANSYS script consists of a mechanical script and a thermal script. The problem is a transient thermal and mechanical problem. The thermal boundary over two seconds is calculated. At the end of two seconds, the end state of the transient thermal problem becomes the load for a separate mechanical calculation. The thermal deformation, ice expansion and

strain are calculated. Once the mechanical calculations are complete, the calculated geometry deformations become the initial geometry for the next 2 seconds of thermal calculations. The ET will shrink due to the thermal expansion coefficient and expanded due to the density change with ice formation. In short, the model updates the geometry after every 2 seconds. The temperature profile becomes a load at every 2 seconds. Expansion and contraction are calculated, and the geometry is updated. Then the model continues the thermal integration.

2.4 Material Properties

Material properties are balanced out in regards to the volume of the two phases. Properties of specific heat were averaged by volume. The amount of collagen, by mass or by volume is very small in comparison to the amount of interstial fluid which is mostly saline which in large part is just water. Specifically, the volume of collagen for 3mg/ml is only 0.2% by volume and 0.3% by mass total ET. The properties used in the calculations include Young's modulus, enthalpy, specific heat, density, and thermal conductivity. All properties are taken for the individual liquid and solid phase then averaged out using the respective volume fractions. The material properties are based on temperature, but are non-linear. The temperature depends on the phase and latent heat dissipation. The latent heat dissipation was assumed to be linear in order to simplify the problem, though in other studies it has been found that the latent heat dissipation is non-linear [14].

2.4.1 Expansion Due To Phase Change

Ansys does not have a simple way of inputting expansion due to phase change. The best way to do this was to include thermal expansion in the thermal expansion coefficient. In order to get the correct thermal expansion we turned to another study that plotted the strain versus temperature [12].] Using the following equation to plot the expected strain through the phase change, which starts at -.5℃ and finishes at -20℃, the strain of the ET can be plotted.

$$\varepsilon^{th} = \int_{T_r}^{T} \alpha dT + \frac{1}{3} e F_w F(T) \qquad \text{Eq. (2.7)}$$

Were

ε^{th}	is the thermal strain
dT	the temperature difference
α	thermal expansion coefficient
e	volumetric expansion associated with tissue water phase change
F_w	water fraction in the system
$F(T)$	fraction of water that is ice
T	temperature
T_r	reference temperature

To find ε^{th} the first and second terms of equation 1 are plotted separately and added together to give the below plot.

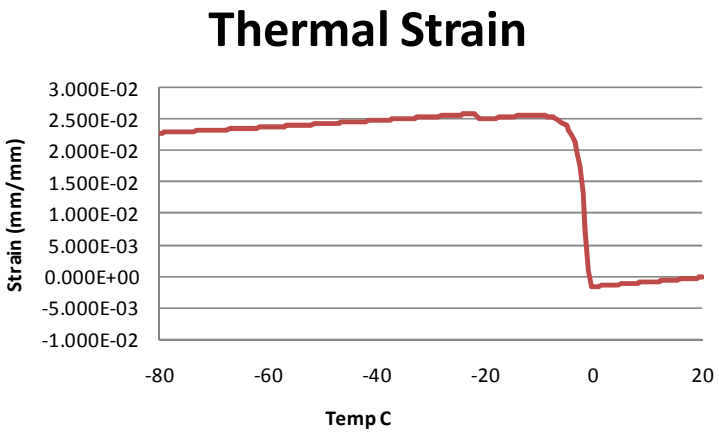


Figure 2.3 Thermal strain plot as a function of temperature.

The thermal strain given in the above figure was the desired strain for the thermal model of the ET. The plotted thermal strain consisted of two separate terms but there was no reasonable way to incorporate the effects of both terms separately in Ansys. In order to incorporate both

terms equation 2.7 would be solved but then the solution of thermal strain would be rewritten to give an effect coefficient of thermal expansion as seen in equation 2.8.

$$\varepsilon^{th} = \int_{T_r}^{T} \alpha_{eff}\, dT \qquad \text{Eq. (2.8)}$$

These new thermal coefficient of expansion would incorporate the effects of both terms in equation 2.7 into a single term that could easily be inputted into Ansys

CHAPTER 3

MATERIALS AND METHODS

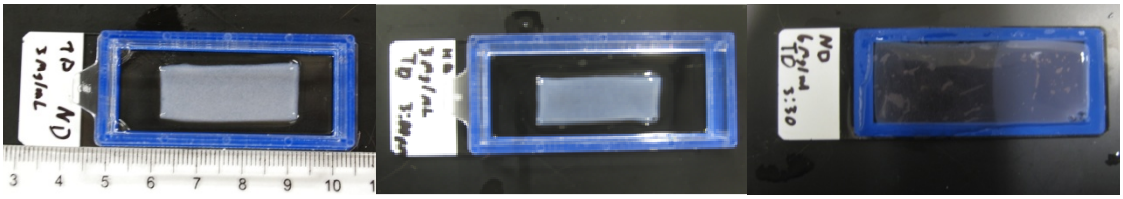
3.1 Cell Culture

The cells used are human fibroblast BR-5 cells. These cells were cultured in a corning flask with culture medium for up to 1 week or when the flask is visibly filled with cells. Cells are cultured in a medium of Advanced DMEM/F1 supplemented with fetal bovine serum (FBS), L-glutamax, and Penicillin/Streptomycin (P/S). 500 ml of Advanced DMEM/F12 is mixed with 50 ml of FBS, 5 ml of L-glutamax and 5 ml of P/S. Cells become attached to the flask. To harvest them, the flask is emptied of all culture medium, rinsed twice with Dulbecco's Phosphate Buffered Saline (DPBS) and then incubated with 2 ml of Trypsin-EDTA for 10 minutes at 37℃. This detaches the cells from the bottom of the flask and 8 ml of the culture medium are added to neutralize the Trypsin-EDTA. The cells suspended in the culture medium are then mixed so that cells are homogenously suspended throughout the culture medium. The cells are then counted using a haemocytometer and inverted microscope at 10x to calculate the amount of cells/ml. The required amount of medium with cells is then pipetted out to get the desired amount of cells and then run through a centrifuge to separate the cells from the medium. The medium is then cantered out and a pellet of cells is then ready to be mixed with the collagen solution for the creation of an ECM matrix with cells or an engineered tissue.

3.2 Engineered Tissue Preparation

Collagen is prepared by mixing several reagents with rat tail type I collagen. First, 10X MEM, 1.0 N NaOH, Distilled water, 0.1 M Hepes , FBS P/S, and L-Glutamine are mixed together in a centrifuge tube, depending on the desired collagen concentration and kept cool by keeping the centrifuge tube in an ice bath. These are then mixed with the pellet of cells mentioned in the previous section. Finally, rat tail collagen is mixed in with the cells and

reagents. The mixture is carefully mixed while being continually kept cool. If the collagen becomes too warm, polymerization will happen prematurely and become unsuitable for casting the gel or ET slide. The mixture is then poured onto a micro-slide and incubated for 1 hour at 37℃ so that mixture can polymerize and become a slide of ET. At this point culture medium is added to feed the cells and the edges of the gel are detached from the slide. The gel is allowed to incubate for an additional 23 hours at 37℃. With cells present, the gel will shrink as the cells anchors to and contracts the collagen fibrils. This can be visualized below.



(a) (b) (c)

Figure 3.1 Casted engineered tissue after 24 hours of incubation a) ND with 3 mg/ml collagen concentration b) HD with 3 mg/ml collagen concentration c) partially frozen ND with 6 mg/ml collagen concentration

3.3 Sample Freezing Procedures

The ET slides are frozen using two stage solidification. Both stages are cooled using gaseous liquid nitrogen. The first stage is cooled to -20℃ and the second stage is cooled to 4℃. Both stages are wiped with 100% ethanol to prevent ice formation and ensure a good contact between the micro slide and cooling stages. The ET slide is taken from the incubator and placed on the 4℃ stage for 5 minutes to allow it to cool to equilibrium. The ET slide is then aligned so that one edge is over the -20℃ stage. A motorized arm then slowly moves the ET slide over to the -20℃ stage so that the ET slide experiences a cooling rate of 5℃/minute. Freezing will usually be allowed to continue until half the ET slide is frozen.

3.4 SEM Preparation Protocol

Once the ET slide has been frozen, the ET slide is then carefully rinsed in distilled water to remove any debris and phosphates present in the ET. After rinsing the ET slide is placed in 2% tannic acid for 1 minute which fixes the ET to fix the ECM structure. The ET is

then rinsed again three times in distilled water and then cut to 3mm diameter disks. The small disks are then placed inside a multi-well plate and stained in 2% uranyl acetate and incubated at room temperature for 15 minutes. The disks are then placed on the Quantomix capsule membrane of the Quantomix capsules along with 10 micro-liters of a buffering solution and finally encapsulated.

3.5 Image Acquisition and Analysis

The engineered tissue, after being prepared, is imaged inside the QX-302 capsules using SEM in combination of BSE detector. The samples are imaged at 15kV with a spot size of 35-45% and contrast turned up as high as possible for the best images. The working distance is optimal at about 9.5 mm and images are taken at 2000x. To process the images obtained the program Image-J is used.

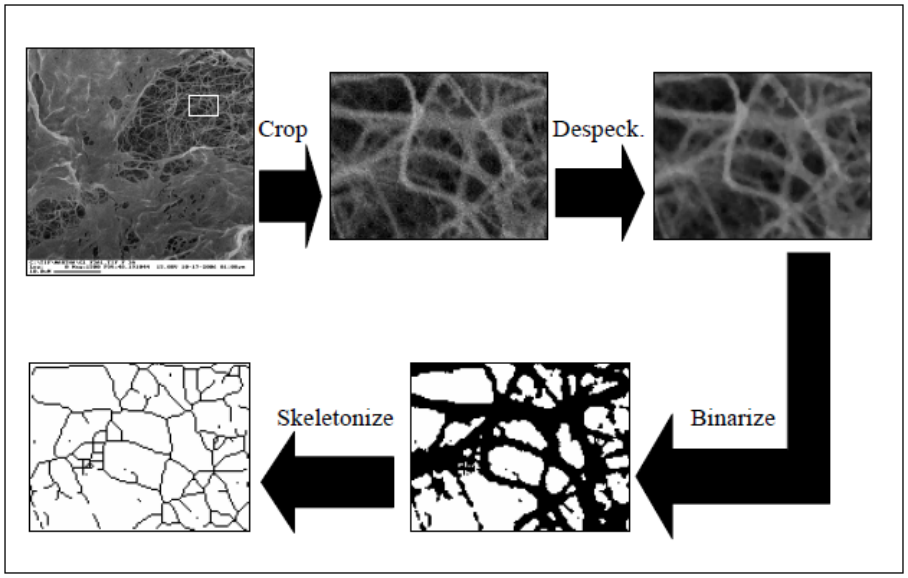


Figure 3.2 Image analysis protocol. Used to evaluate the FA MVA from SEM images: The cropped area is highlighted. Miller et al., 2006

Images are then post processed in Image J, where they are cropped despeckled, made binary, skeletonized and then measured for mean void area, and fibril thickness. In order to make the images binary, a script code is used to make sure the same process is used every time. First the image is despeckled. After which the contrast is increased by 2% in Image J. Next lightest

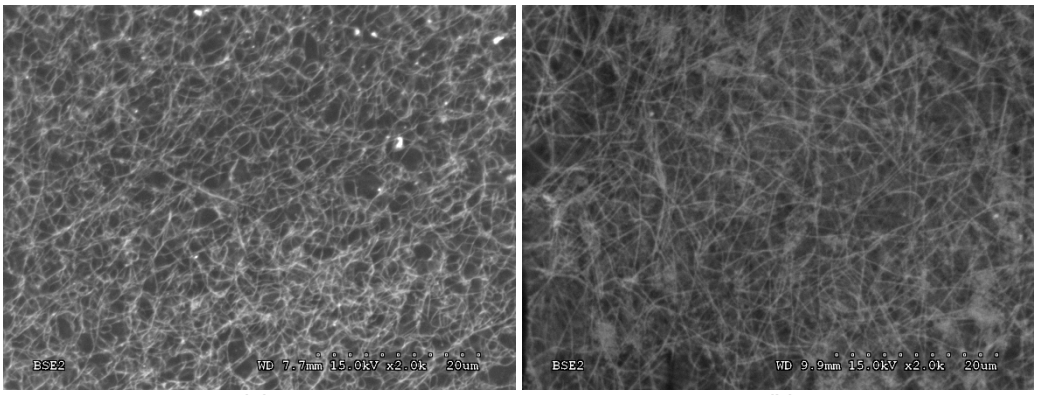
contrast is equalized so that the brightest pixels are made completely white and the darkest pixels are made completely black. All pixels in between are spread out on the gray scale and equalized so that the white pixels have a value of 0 and black pixels and white pixels have the value of 255. All other pixels are assigned a value in between according to their brightness intensity. Finally a threshold is set so that all pixels with values greater than 114 are made black and pixels with values less than 114 are made white. To skeletonized the image is done automatically by Image J itself and has no further variables.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 SEM Images

Using the Quantomix capsules and having frozen/thawed and unfrozen region's samples, images are then taken, using SEM, of three types of engineered tissue (ET). Of the three types, each will have an unfrozen and frozen/thawed section that will be viewed. In this section the differences between different collagen and cell densities and frozen/thawed and unfrozen regions will be observed. The two variables of ET, collagen density and cell density are adjusted in three different ways which give the three types of ET in this study. The decided ET types to be studied are 1) 3mg/ml of collagen with 200,000 cells per ml of engineered tissue, 2) 3mg/ml of collagen with 400,000 cells per ml of engineered tissue and 3) 3mg/ml of collagen with 200,000 cells per ml of engineered tissue. It was decided to call 200,000 cells per ml the nominal cell density (ND), with 400,000cells per ml being the high cell density (HD).

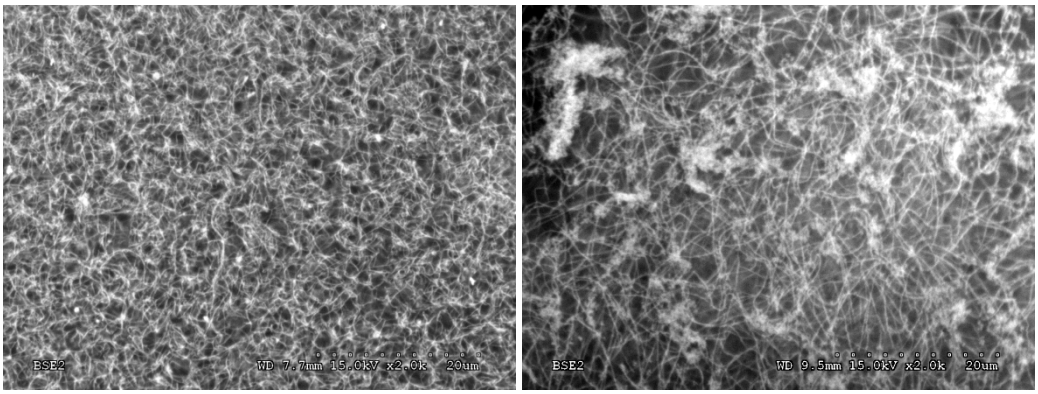


(a) (b)

Figure 4.1 A 3mg/ml density of collagen with a nominal density of cells. These images are at 2000x magnification using BSE and a uranyl acetate staining solution. a) Unfrozen b) Frozen

Collagen as mentioned before under high magnification appears as thin long fibrils which make up the ECM of the tissue. Observing some of the differences between the frozen/thawed and

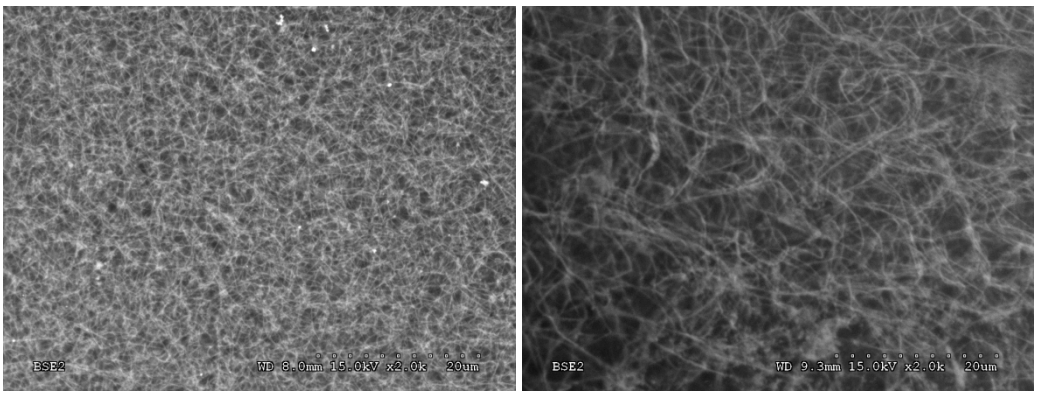
unfrozen regions, it can be seen that the fibrils in the unfrozen section are very uniform and closely compacted. In the frozen section a cloudy material sometimes appears with the fibrils.



(a) (b)

Figure 4.2 A 3mg/ml density of collagen with a high density of cells. These images are at 2000x magnification using BSE and a uranyl acetate staining solution. a) Unfrozen b) Frozen

Comparing figure 4.1 with figure 4.2 it's seen that in the 3mg/ml and HD engineered tissue, the ECM is much more compact in the unfrozen regions, but in the frozen/thawed regions, the collagen fibrils themselves seem to have more space between them. This would have us predict that the mean void area should increase. The calculated results appear later in this study.



(a) (b)

Figure 4.3 A 6mg/ml density of collagen with nominal density of cells. These images are at 2000x magnification using BSE and a uranyl acetate staining solution. a) Unfrozen b) Frozen

The 6mg /ml collagen density ND appears to be similarly compact to that of the 3mg/ml collagen HD in the unfrozen region, but as seen in the 3mg/ml and high cell density tissue, the F/T regions have undergone similar changes to the ECM.

4.2 Fibril Area & Mean Void Area

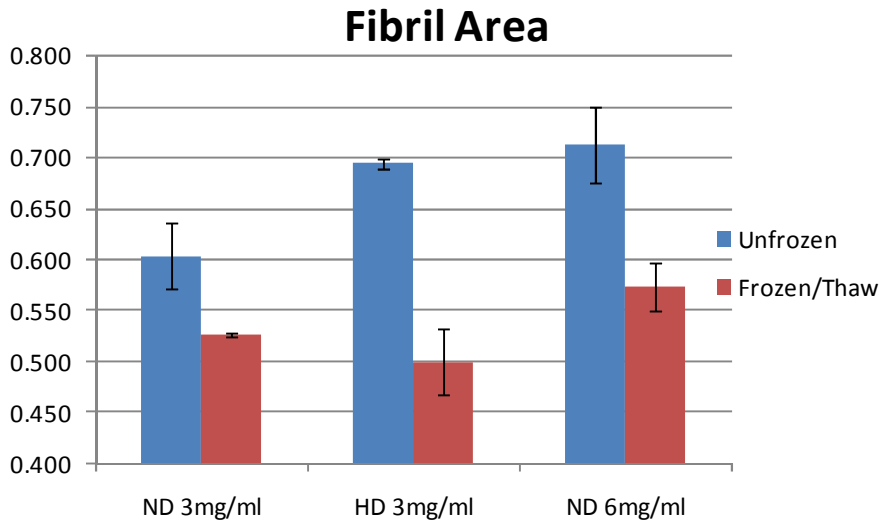
The fibril area is calculated as outlined in section 3.5. A cropped section of the SEM image is binarized and the number of black pixels counted by Image J using the histogram tool and recorded as the fibril area (FA) after converting the number pixels into normalized number between 0 and 1. The FA is what allows us to know the amount of collagen is in a 2-D area. The process of binarizing only accounts for the collagen fibrils at the top layer. Fibrils under the top layer appear as a darker shade and are ignored by setting the threshold value before binarizing the image so only the brightest layer is part of the FA calculation. Numerous crops are cropped then binarized and their subsequent histograms are observed to find the mean FA. To compare data, the standard deviation (SD) and percent standard deviation (% SD) were also calculated. The student-t test was calculated with equal variance assumption. The first tail p-value of the student t-test is assumed to be significant as long as its value is below .05 or less than 5% chance that one crop from the unfrozen sample group would overlap with the distribution of the frozen/thawed sample group. The statistical data is then compared between the types of engineered tissue and between the frozen/thawed and frozen regions. The final table of results can be seen in table 4.1.

Table 4.1 Fibril Area and Mean Void Area Calculation Results.

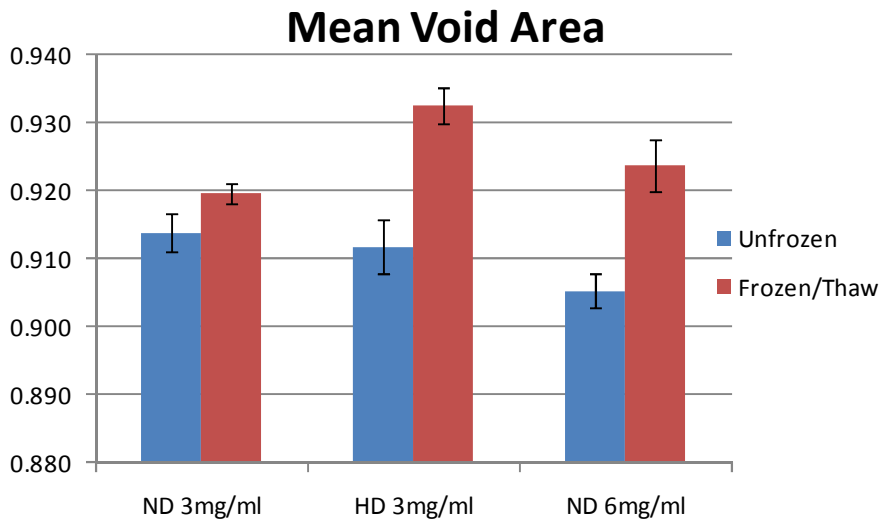
	FA	FA SD	FA %SD	FA P-value	MVA	MVA SD	MVA %SD	MVA P-value	# of Crops
ND 3mg/ml Unf	0.603	0.032	5.07	4.94E-12	0.914	0.003	0.30	0.0773	15
ND 3mg/ml F/T	0.527	0.002	0.34		0.920	0.002	0.16		15
HD 3mg/ml Unf	0.694	0.005	0.65	2.31E-07	0.912	0.004	0.44	2.68E-07	15
HD 3mg/ml F/T	0.500	0.032	6.01		0.933	0.003	0.28		15
ND 6mg/ml Unf	0.712	0.037	4.96	9.00E-06	0.905	0.003	0.29	1.48E-07	15
ND 6mg/ml F/T	0.573	0.023	3.90		0.924	0.004	0.41		15

Just because the fibril area increases or decreases does not mean there are more or less fibrils unless the diameter of the fibrils is unchanged. In order to account for any fibril diameter change the mean void area (MVA) is calculated by taking the binary image and skeletonizing the fibrils. If the fibrils diameter has decreased and number of fibrils is the same, the

skeletonization in the frozen/thawed and unfrozen regions could have given similar numbers of pixels. If the number of fibrils has decreased but diameter has increased, the MVA calculation should help reveal these changes.



(a)



(b)

Figure 4.4 Plot of statistical results for SEM images: a) fibril area with standard deviation as error bars b) Mean void area with standard deviation making up the errors bars.

The results are consistent with the visual observations and statistical data shows the results are consistent. From this data we can see the fibril area decreases after freezing and that the mean

void area increases. With fibril area decreasing and mean void area increasing it's assumed that the voids between fibrils have grown in size and less collagen fibrils are present after freezing. To make table 4.1 easier to understand, the results are graphed in figure 4.4.

4.2.2 Discussion of FA and MVA

The fibril area decreases and the MVA increases after the freeze/thaw process. This is probably due to the expansion of the ice crystals in between the collagen fibrils. As the interstitial fluid of the ET freezes it will expand and cause the MVA to increase and at the same time pushing collagen. The possible reason for the change in the mean void area is the expansion of the ice crystals in between the fibrils of collagen. As the interstitial fluid of the ET freezes it will expand and cause the MVA to increase.

4.3 Fibril Diameter

Using the same cropped images used to find FA and MVA, the fibril diameter will be examined. The calculation is simple using Image J. Every cropped image is examined for thee clear fibrils. Using the line tool, a line is drawn across the diameter of the fibril. The fibril diameter is given in pixels in Image J. Using the scale bar to correlate pixels to nm, the diameter of the fibrils is calculated.

Table 4.2 Average Fibril Diameter Calculation Results.

	Average(nm)	Stand Dev	%Stand Dev	P-value	# Measured
ND 3mg/ml Unf	177.47	24.08	11.95	1.58E-05	16
ND 3mg/ml F/T	224.47	29.04	11.46		16
HD 3mg/ml Unf	169.50	15.55	8.40	3.5E-10	16
HD 3mg/ml F/T	253.53	33.85	11.78		16
ND 6mg/ml Unf	157.90	15.58	8.98	1.26E-10	16
ND 6mg/ml F/T	239.10	32.74	12.04		16

The data in table 4.2 is plotted below in figure 4.5 to give a better visual comparison of what freezing and thawing effects have on the collagen fibrils. Using a similar calculation procedure for FA and MVA, the average pixel diameter is found along with its standard deviation. The standard deviation is then converted into a percentage to give an idea of the

significance of the deviation. When comparing the fibril diameter results it can be noted that he diameter has been increased for all ET types between the unfrozen and frozen/thawed regions. Also to be observed is that the standard deviation also has increased as the ET's are frozen/thawed, meaning the diameters are not uniformly thicker after freezing/thawing.

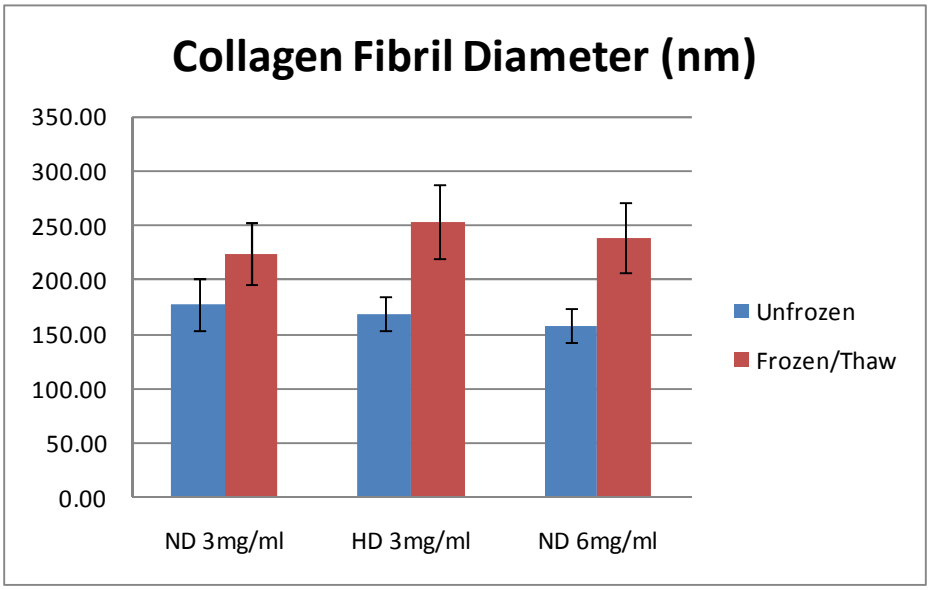


Figure 4.5 Plot of collagen fibril diameter along with standard deviation error bars. Unfrozen sections and frozen sections included

4.3.1 Discussion of the Fibril Diameter

The diameter of fibrils is a bit curious. Fibrils are self assembled proteins in a triple helix called a tropocollagen [16]. Tropocollagens aggregate together to make a single collagen fibril. For there to be an increase in diameter of the fibrils could mean the number of tropocollagens in a single fibril have increased, or the bond between tropocollagens has given way causing spaces between them. Perhaps it is possible for one collagen fibril to be broken down and the individual tropocollagens could bond to other collagen fibrils that avoided being broken down after freeze/thawing. Perhaps the fibrils diameter increase was caused by bonds between tropocollagen bonds somehow being weakened so that spaces where formed during freezing and were not repaired during thawing. More study on this subject would be warranted.

4.4 Comparison to Previous Study

As shown in a previous study by Jeffrey Miller [6] at the University of Arlington many of the above results follow the same trend. Most notably is that diameter of the fibrils increases after the F/T process. Also the fibril area decreases after F/T and MVA increase after F/T. Also noticeably the same is that the fibril diameter change experienced by collagen frozen to -20℃ was not significantly different. This meaning that the presence of cells did not change the ratio of fibril diameters between unfrozen and F/T samples. The average FA and MVA were quite different between the two studies. This is probably due to the high compaction of caused by the fibroblast cells. These cells have greatly compacted the collagen so that much more fibril material is in the same window than in the previous study.

4.5 Techplot Post Processing

The modeled solutions taken from Ansys were strain, deformation and temperature. In order to get the necessary calculations of deformation rate, strain rate and dilatation it was necessary to do some post processing. A powerful FEA post processing tool was found in the program Techplot. Using Techplot, simple calculations such as finding rates and converting units is easily done.

4.5.1 Freezing Front Location

Using the material properties as calculated before it was found that the location of the freezing front or freezing interface was moving much faster in Ansys output than in the experimentation done in a previous study [15]. This can be seen in figure 4.6.

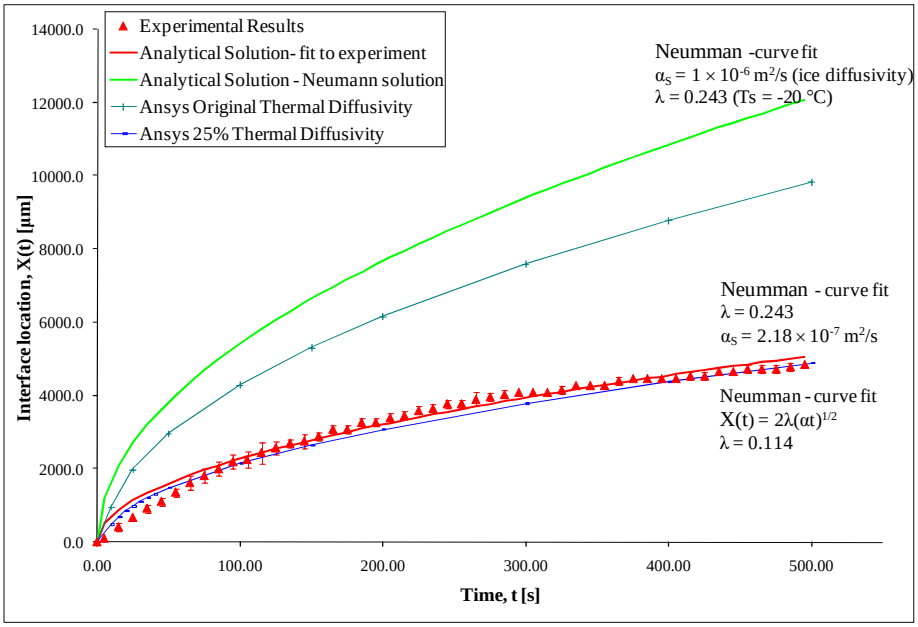


Figure 4.6 Curve fit of Ansys solutions to experimental solutions for the interface location. The light green curve represents the analytical solution using thermal properties of pure ice. The Ansys solution is seen in the darker green. The Neumann solution is fitted to the experimental results (red dots) by first adjusting lambda then thermal diffusivity (red line). The blue line represents the Ansys solution with an effective thermal diffusivity.

Also the prediction of freezing front location according to the Neumann solution also over predicted the speed of the freezing interface according to the properties that are known for the ET which mostly consists of water. The Neumann solution is a simplistic approximation but can give accurate results. The two major variables in the Neumann solution are, α ,the thermal diffusivity and, λ , a constant that must be determined from the Stefan Number which relates the boundary temperature, initial conditions and phase change parameters to the Neumann solution. The equation for the Neumann solution is seen in equation 4.1

$$X(t) = 2\lambda\sqrt{\alpha t} \qquad \text{Eq. (4.1)}$$

This equation only predicts the freezing front location which is a set temperature. For this model the freezing interface is considered to be the point at which the FE model is at -0.5℃. There has not been much study on the thermal conductivity of ET so as to suggest the thermal properties that have been collected for this study may not be the best. If the Neumann solution

is not accurate to the experimental data, there are only two variables to change to get the curve to fit. λ is dependent on the boundary conditions so it's desired to keep that the same to have comparable results. Therefore, it was decided to change the thermal diffusivity, α, to an effective thermal diffusivity to match the Ansys and Neumann solutions to the experimental results for freezing interface location with respect to time. For this study it was found that 25% of the original thermal diffusivity provided to Ansys gave a solution that fit very closely to the curve fitted Neumann solution and experimental data as seen in figure 4.6. The reason for the discrepancy between the original FE model and experimental results could be due to the experimental set up. The ET in the experiment may have added thermal resistance and mass due to the glass slide that it rest upon as it passes over the two freezing stages. The glass slide which the ET rest upon is also thick, 1.2mm, when compared to the 2mm thick ET. The glass slide is also not a highly conductive material and would also add to the discrepancy between the modeled results using the original thermal properties and the experimental results. Also contact resistances between the glass, stage and ET itself could add to the resistance and further delay the experimental results to achieving the ideal results calculated by Ansys. These real world conditions are likely the cause of the effective thermal diffusivity only being 25% of the original model. Smaller dissimilarities between FE model and experimental setup could add to that such as the unaccounted convection to room temperature. It's also possible that the warm boundary in the FE model and the experiments aren't the same, it could also add to the discrepancy, but it's been shown using the Neumann solution that the differences between the warm 4℃ and 20℃ are small as long as the end free ze temperature is the same. To achieve the matching of interface location between FE model and experimental results the FE model used an effective thermal diffusivity that was achieved by using 25% of the original thermal conductivity. The following results reflect the effective thermal conductivity.

4.5.2 Deformation Rate and Dilatation

Using Techplot the deformation rate and strain rate were obtained for the x, y, and z directions. In order to do this the deformation and strain results were taken from Ansys.

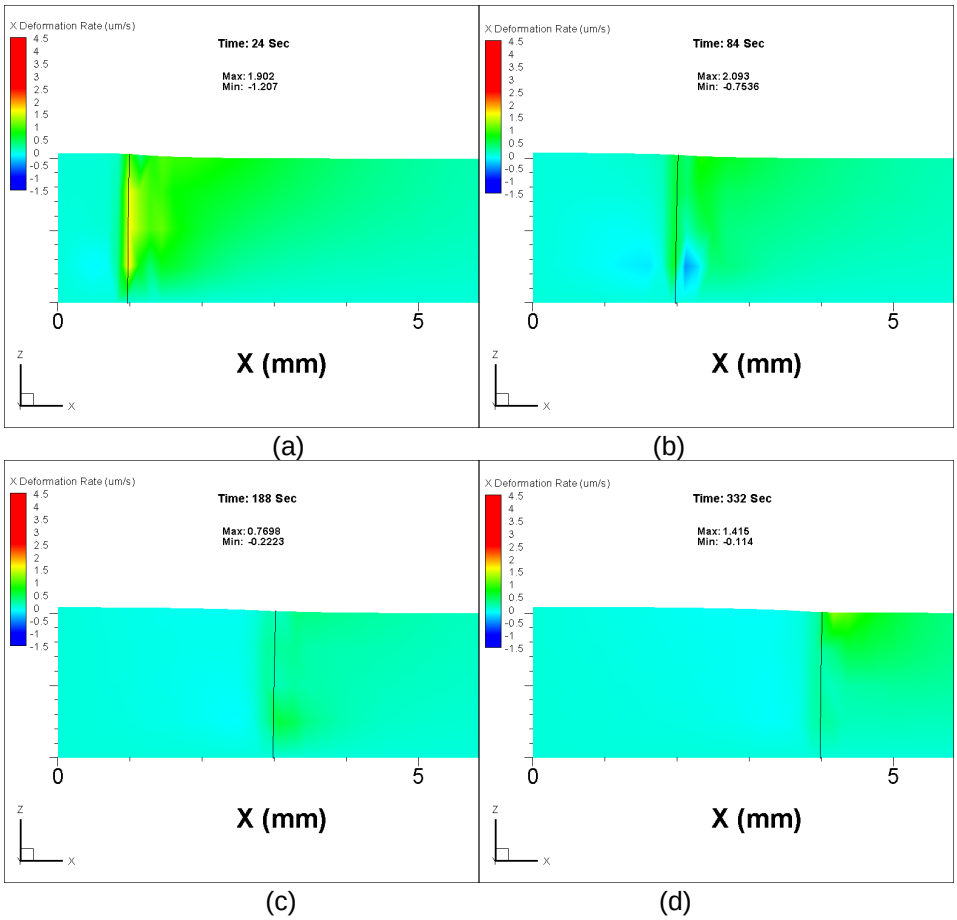


Figure 4.7 Deformation rate for 25% thermal diffusivity in the x-direction when the freezing front (black line=-0.5℃) is at a) 1mm b) 2mm c) 3mm d) 4mm

Using those results, the two new variables were made by using the following formula in Techplot.

$$U_n = \frac{D_{n+1} - D_{n-1}}{2 \cdot \Delta t} \qquad \text{Eq. (4.2)}$$

U is the variable rate to be calculated, D is the Ansys variable output, n is the time step number and Δt is the size of the time step. Some of the results can be seen in figure 4.6 and 4.7.

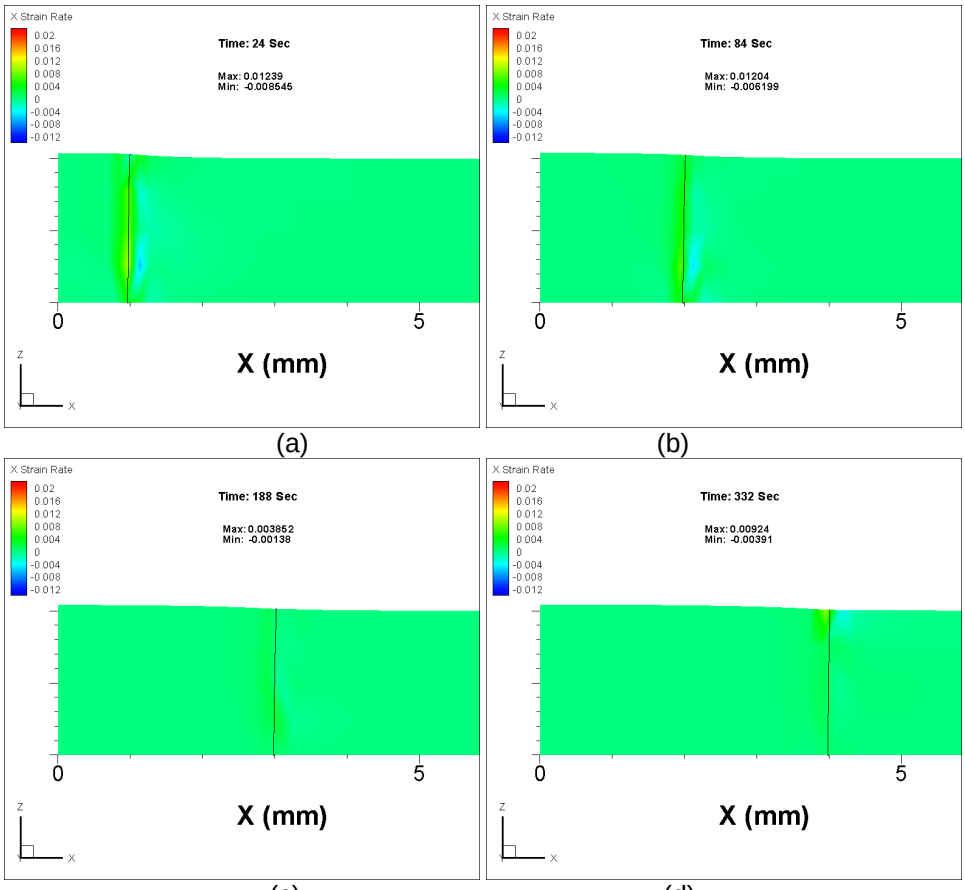


Figure 4.8 Strain rate for 25% thermal diffusivity in the x-direction when the freezing front (black line =-0.5℃) is at a)1mm b) 2mm c) 3m m d) 4mm

Since the model is actually a 3-d model, figure 4.9 gives an idea of the 3-d impact by showing the 3-d vector plot of the deformation rates in the x, y, and z direction for the effective diffusivity material properties. Using Techplot to extract data from the Ansys files results are exported into Excel. The following model results were obtained by running the Ansys script and then using Techplot to post process such information as dilatation, deformation rates, and strain rates.

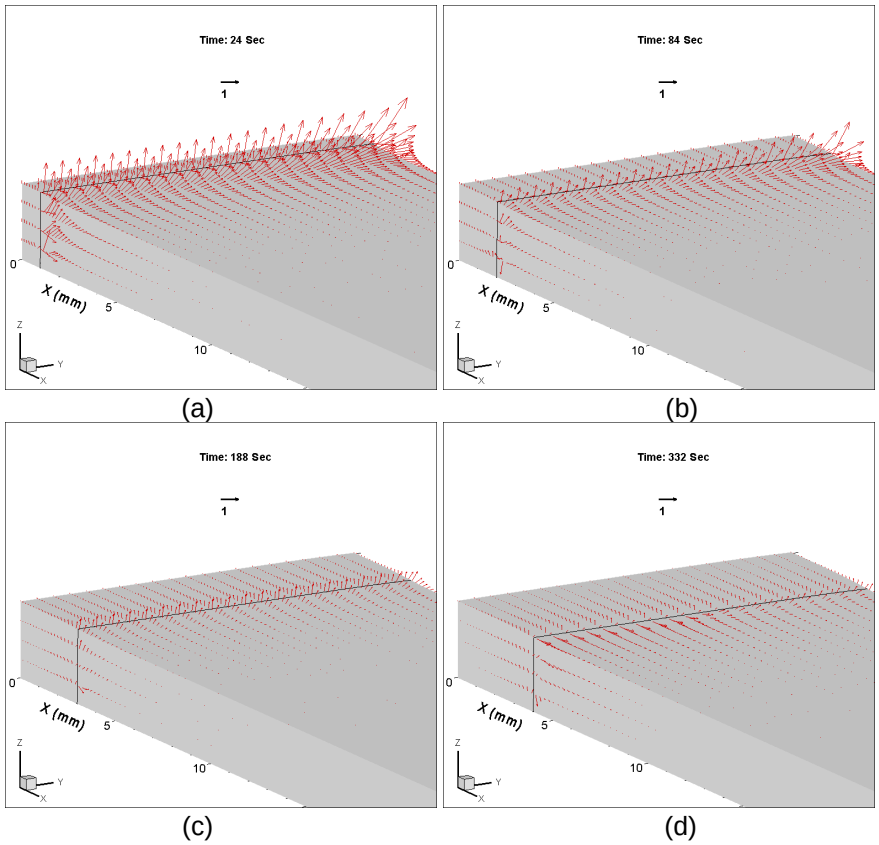
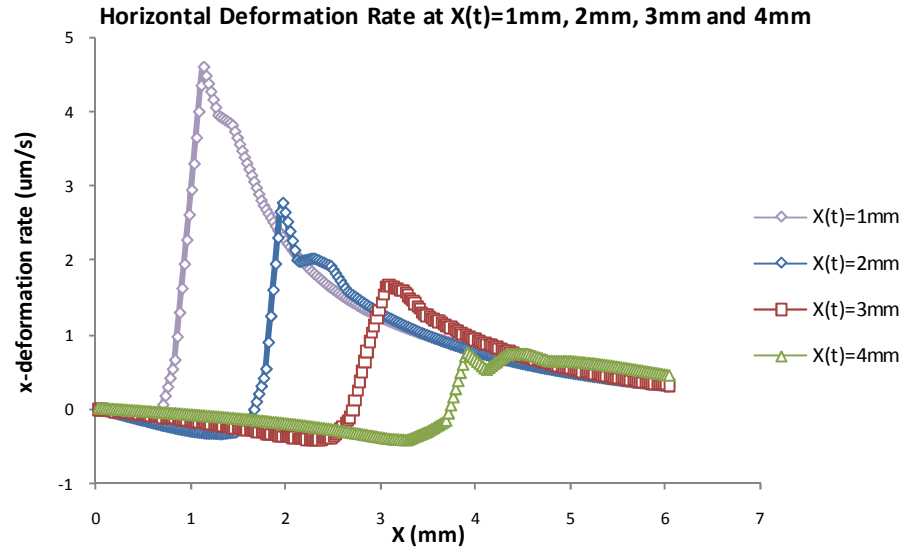
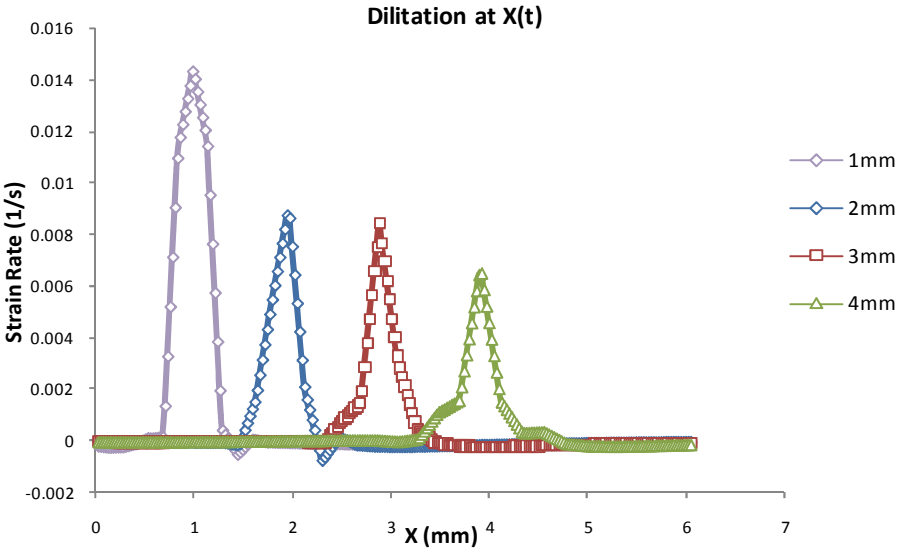


Figure 4.9 The 3-D vector plots for deformation rates at interface locations for a) 1mm b) 2mm c) 3mm d) 4mm

From Techplot data was extracted from times where the freezing interface passed through several locations. The results in figure 4.10 show data taken from along the line of symmetry at a z-height of 1mm in a 2-d plot. This location was chosen since the surface of the model deformed at a greater magnitude which made it difficult to get a single line of elements. The several lines in the results of these plots correlate to the different times to which the freezing interface passed through the x location stated in the legend. When the freezing interface is at 1mm data is extracted as well as at 2mm, 3mm, and 4mm. The correlating times to those locations can be seen in figure 4.6 or in appendix D

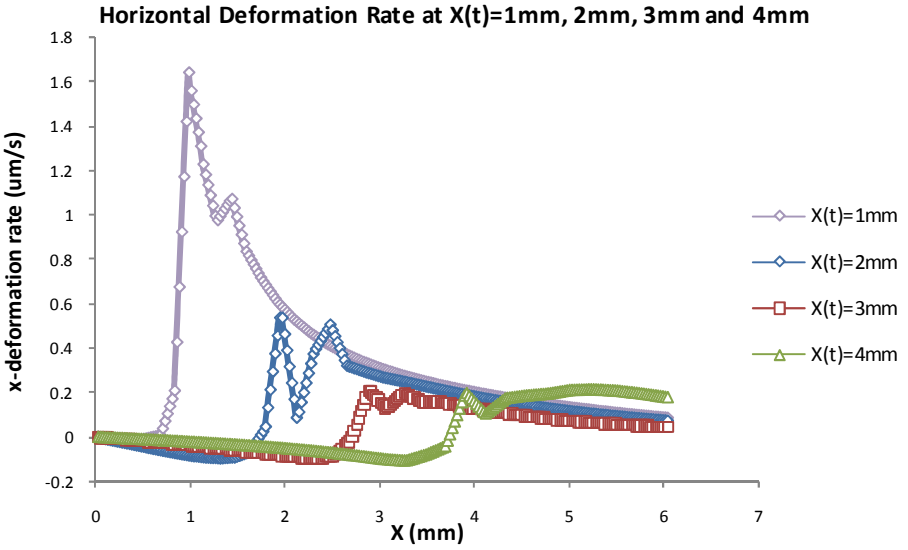


(a)

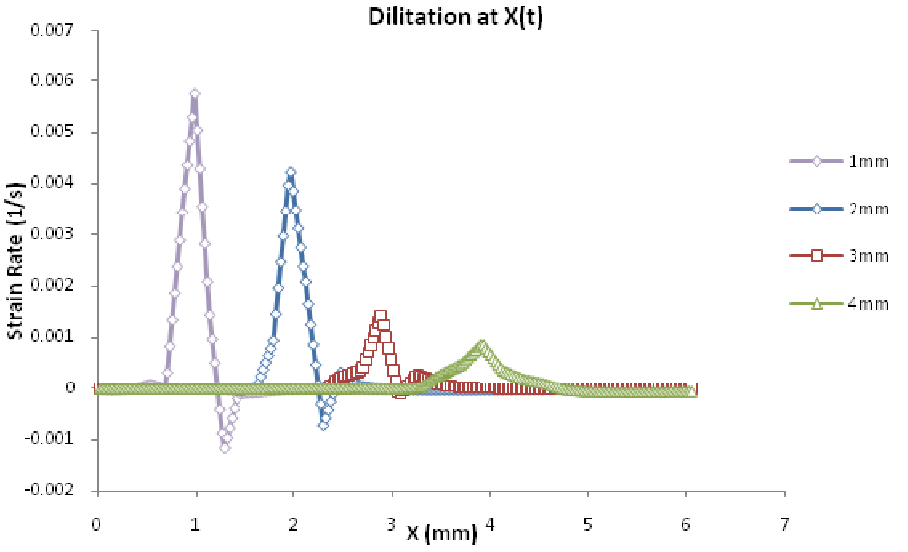


(b)

Figure 4.10 Post processing results at location z=1 mm and y=0 mm of model and 100% thermal diffusivity. a) Deformation rate along x-axis b) Magnitude of dilatation as freezing front passes through 1, 2, 3, and 4 mm.



(a)

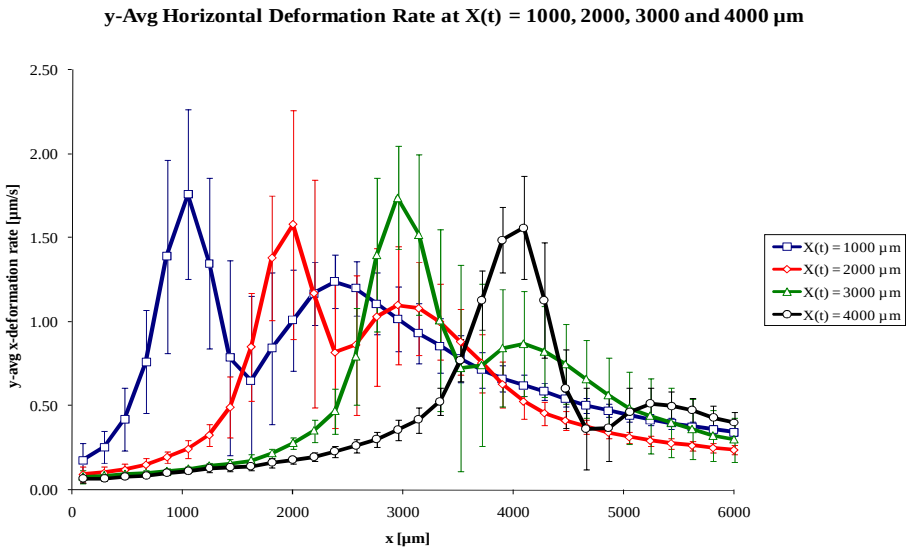


(b)

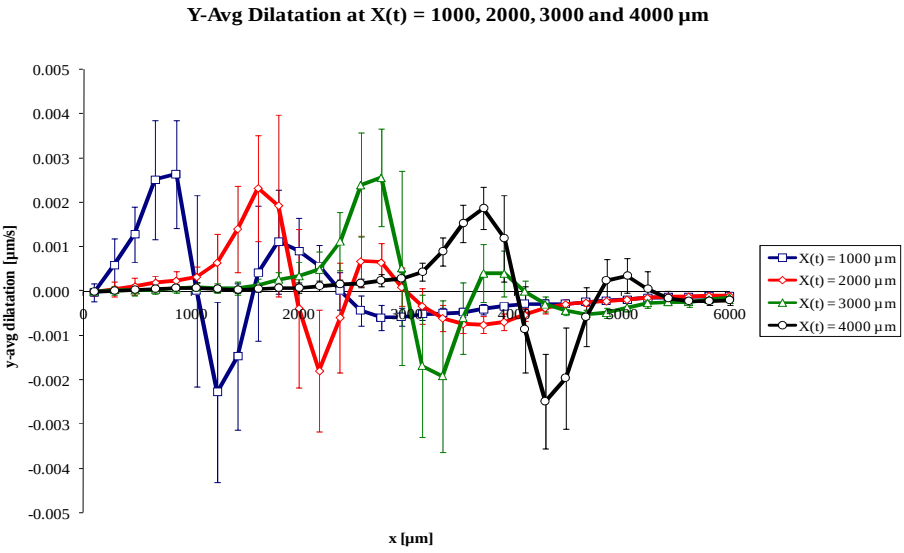
Figure 4.11 Post processing results at location z=1.0 mm and y=0 mm of model and 25% thermal diffusivity a) deformation rate along x-axis b) magnitude of dilatation as freezing front passes through 1, 2, 3, and 4 mm.

4.6 Discussion of Comparison of FE Model Results and Experimental Results

When comparing the modeled results to the experimental results found in a separate study [15] it can be seen that the trends are similar as the horizontal deformation rises then falls and rises again. This is common for all the horizontal deformations.



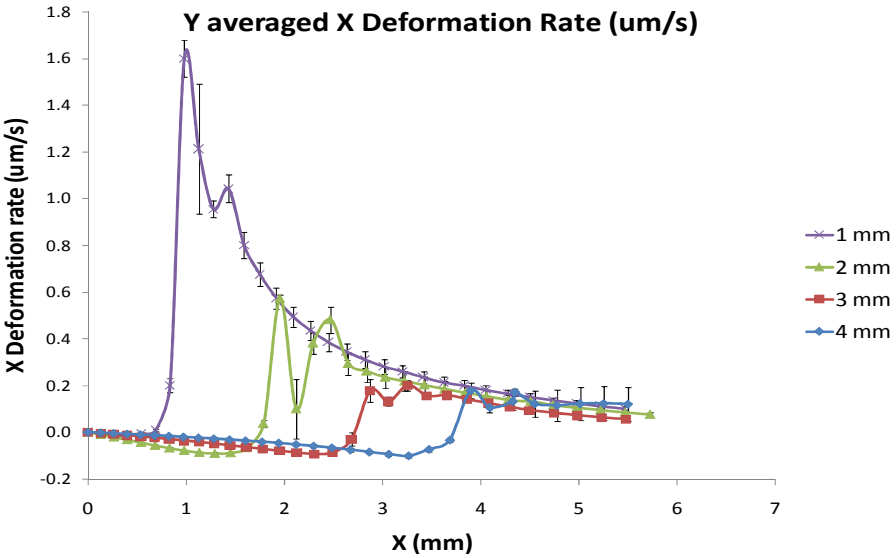
(a)



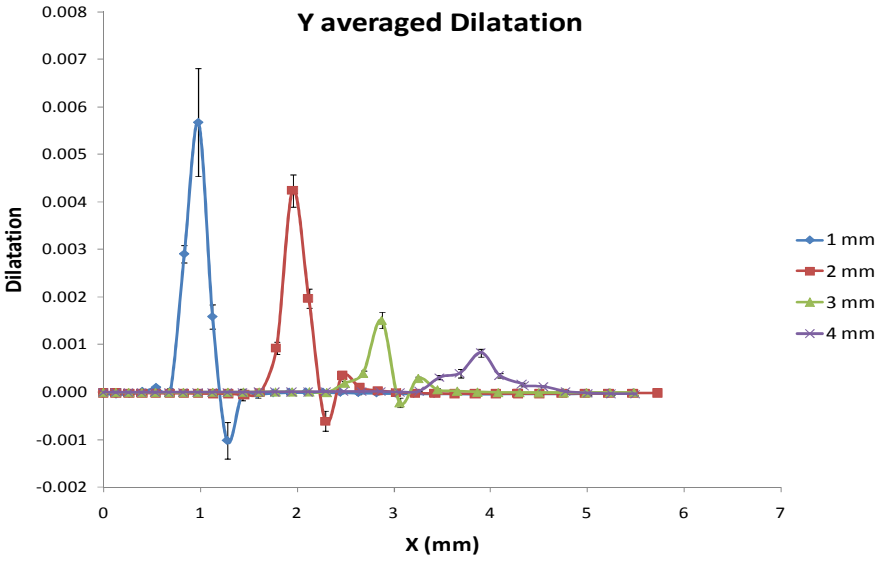
(b)

Figure 4.12 Experimental results of a) deformation rate along x-axis b) magnitude of dilatation as freezing front passes through 1, 2, 3, and 4 mm. Teo et al. 2009

The dilatation is a little less consistent with the modeled results but the magnitude is similar. There isn't as much negative dilatation at the location of the freezing front though some is present especially at the early times at 1mm and 2 mm for results using the effective thermal diffusivity and the original thermal diffusivity. For the comparison with the experimental results to be made, an averaging across the y axis along the z=1 mm.



(a)



(b)

Figure 4.13 Post processing results of Y- averaged variables at location z=1.0 mm and y=0 mm of model and 25% thermal diffusivity a) deformation rate along x-axis b) magnitude of dilatation as freezing front passes through 1, 2, 3, and 4 mm.

CHAPTER 5

CONCLUSIONS

Observing the microstructural changes of the ET due to freezing was done in hopes of understanding what causes the mechanical properties of the tissue to change. In viewing the ET before and after freezing the following conclusions and observations were made.

1) Freezing of ET causes of a decrease in the average fibril area.
2) The diameters of the fibrils were increased after the freeze thaw process.
3) Mean void area increased
4) Freezing interface location can be predicted using thermal modeling in Ansys for this experimental setup

These three major observations would leave one to believe that the freezing process not only causes microstructural changes due to ice formation increasing the porosity but actually changes each individual fibril and increases its size. From above results we can see that adding cells does not change the effects of freezing on the change of fibril diameter. It's also observed that even though F/T can decrease FA and increase MVA, the presence of cells will still greatly increase the overall FA and decrease the overall MVA as opposed to when cells are absent. The cause of individual fibrils changing would warrant greater study. Also, a closer examination of the thermal and mechanical properties of the ET could increase the accuracy of the thermo-mechanical model. What must be addressed are certain limitations of this study. One limitation of the process used was the Quantomix capsules. The ideal situation would be to use and environmental scanning electron microscopy instead of the Quantomix capsules.

Due to cost of such a piece of equipment, this was not possible. Such a piece of equipment has shown to give greater contrast and may make it much easier to visualize ECM and cell interaction without the need of critical point drying and other processes needed for traditional SEM. The model in the study is limited in that many of the original material properties used in this study were taken from other studies and in places where no direct study was made, the material properties of water and collagen had to be balanced out. Further study of the ET's material properties could help improve the model and determine if the effective thermal diffusivity is mostly a result of inaccurate material properties or more because of contact resistances. In the future it's hoped this study can be continued material properties found specifically for the ET's used in the study. Also it would be interesting to study greater the actual cause of the change in diameter of the collagen fibrils. The purpose of this study is to better understand the effects of freezing on the ECM, but still what needs to be understood is the reason for the change in fibril diameter due to freezing temperatures.

APPENDIX A

PROTOCOL FOR CULTURING AND SPLITTING BR-5 FIBROBLAST CELLS

Equipments and reagents

1. Glass pipettes
2. 75 cm^2 corning flasks
3. Advanced DMEM/F12 basal medium (Invitrogen, catalog no. 12634-010)
4. Fetal bovine serum (Invitrogen, catalog no. 16000-044)
5. L-glutamax (Invitrogen, catalog no. 35050-61)
6. Penicillin/Streptomycin (Invitrogen, catalog no. 15140-122)
7. Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen, catalog no. 14040-182)
8. Trypsin-EDTA (Invitrogen, catalog no. 25300-062)
9. DMSO (Sigma-Aldrich, product no. D2650)

Cell culture medium description and preparation

Advanced DMEM/F12 basal medium supplemented with the following components:

- FBS, 10% (v/v)
- L-glutamine, 2 mM
- Penicillin, 100 units/mL
- Streptomycin, 100 µg/mL

1. Warm a bottle of 500 mL Advanced DMEM/F12 basal medium.
2. Thaw the following supplements:
    - FBS, 50 mL
    - L-glutamax, 5 mL
    - Penicillin/Streptomycin, 5 mL
3. By using glass pipettes, transfer the supplements into the bottle of basal medium.
4. Label the bottle with “BR5,” date, and your initials.
5. Store the bottle in the fridge at 4 ℃.

Freezing medium description and preparation

Advanced DMEM/F12 basal medium supplemented with the following components:

- FBS, 90% (v/v)
- L-glutamine, 2mM
- Penicillin, 100 units/mL
- Streptomycin, 100 µg/mL
- DMSO, 10% (v/v)

1. Warm the following reagents:
    - Supplemented cell culture medium
    - FBS, 25 mL
    - DMSO, 2 mL
1. Add 20 mL supplemented cell culture medium into a 50-mL microtube.
2. Transfer 17 mL FBS and 2 mL DMSO into the tube.
3. Label the tube with “BR5 freezing medium,” date, and your initials.
4. Store the tube in the freezer at -20 ℃
    1. Secure cap and incubate the new flask.
    2. Collect the rest of the aliquot in the old flask into 15-mL microtube for experiment.

    Note:
    - BR5 has a population doubling time of approximately one week. Please change the cell culture medium and split the cell culture correspondingly.
    - To achieve the highest cell densities, the culture medium should be changed every day as the culture approaches confluence.

- 1st-passage BR5 is capable of 20-25 population doublings, and thereafter the cells tend to lose their capability to proliferate.
- Please see the attached log sheet at the side of the cryotank for more information about the passage number of different BR5 cryotubes.

References

Rhee S, Jiang H, Ho CH, Grinnell F.
Microtubule function in fibroblast spreading is modulated according to the tension state of cell-matrix interactions.
Proc Natl Acad Sci U S A. 2007 Mar 27; 104(13): 5425-30.

Han B.
SOP for Cell Culture Media Preparation. July 28, 2006.

APPENDIX B

PROTOCOL FOR CASTING ENGINEERED TISSUE EQUIVALENTS

SOP for Tissue Equivalent Construct Preparation (with cells)									
(1.0 N NaOH)	Filename: Collagen SOP Date: 6-2-08								
	IMPORTANT NOTE: CHANGE CONCENTRATIONS ON BOTH SHEET 1 AND 2								
I. Materials									
	Final Collagen Concentration:			3	(mg/mL)		Initial Col. Conc.	8.37	mg/mL
		Final volume of collagen solution (in mL)							
		1	2	3	4	5	6	7	8
	Location	**Amt. (µL)**	**Amt. (µL)**	**Amt. (µL)**	**Amt. (µL)**	**Amt. (µL)**	**Amt. (µL)**	**Amt. (µL)**	**Amt. (µL)**
10X MEM	Fridge	100	200	300	400	500	600	700	800
Sterile 1.0 N NaOH	Fridge	8	16	25	33	41	49	58	66
0.1 M Hepes	Fridge	30	60	90	120	150	180	210	240
P/S	Freezer	1	2	3	4	5	6	7	8
L- Glutamine	Freezer	10	20	30	40	50	60	70	80
FBS	Freezer	60	120	180	240	300	360	420	480
Sterile dH2O	Fridge	432	865	1297	1729	2162	2594	3026	3459
Rat Tail Collagen	Fridge	358	717	1075	1434	1792	2151	2509	2867
	TOTAL	1000.00	2000.00	3000.00	4000.00	5000.00	6000.00	7000.00	8000.00

II. Preparation

A. (2) 5 mL pipettes
B. 1000, mL, 250 mL and 20 mL pipette tips
C. Chamber slides
D. 15 mL centrifuge tube
E. Cell culture medium (cell-type dependent)
F. Type I High Concentration Collagen
G. Container with ice cubes

III. Procedure: for 2 mL sample

NOTE: Keep NaOH and dH2O on ice until use; carry out procedures in hood when possible

Warm bath to 37 deg. C; put on gloves; spray with ethanol

A. Warm MEM and Hepes to ~ 37 deg. in waterbath

1. Thaw P/S, L-Glutamine, and FBS in waterbath; WATCH MOUTH OF TUBE. DON'T CONTAMINATE

B. Spray inside bottom of hood with ethanol and wipe
C. Prep pipette knobs to correct sucking amount
D. Retrieve tupperware bowl, spray with ethanol and wipe, put ice in it, put it in hood, put 15 mL centrifuge tube in it
E. Retrieve, spray with ethanol, and wipe down NaOH and dH2O and place them in the bowl of ice in hood

1. Retrieve, wipe down, and spray with ethanol the Hepes, P/S, FBS, and L-Glut and place in hood

F. Retrieve, wipe down, spray with ethanol, and wipe down MEM, then add to the chilled centrifuge tube (in hood) with 250 mL pipette tip
G. Add sterile, ice cold NaOH to MEM (in hood) with 250 mL pipette tip
H. Add Hepes, P/S, L-Glutamine, and FBS
I. Add sterile, ice cold dH2O (in hood) with 1000 mL pipette tip
J. Add solution to washed cell pellets (in 15 mL centrifuge tube)
K. Mix the contents of the tube by quickly pipetting several times

within one minute (avoid bubbles) and hold in ice (practice needed); use 5 mL pipette.

L. Prepare the chamber slide or petri dish (retrieve, mark, and open), new 5 mL pipette, and pipette knob while the tube is on ice
M. Re-spray hands with ethanol, retrieve, spray with ethanol and wipe down collagen. Put on ice in hood.
N. Add collagen (avoid bubbles) with 1000 mL pipette tip and mix using 5 mL pipette from J.
O. Dump the mixture into chamber slide or petri dish using the same 5 mL pipette
P. Place the solution in the incubator and allow to gel at 37oC for 30 min.
Q. While in incubator, refill ice tray and put in freezer; retrieve, spray with ethanol, wipe down, and put PBS in hood

Put MEM, collagen, NaOH, and dH2O back in fridge

R. When gel is ready, add 2 mL culture media to cover the whole gel with 2 mm thickness (for chamber slides)
S. Place in incubator until experiment

NOTE: Check culture medium thickness periodically to avoid gel dehydration

Also, be sure to remove culture media from surface of gel before freezing, fixing, etc.

NOTE: Keep NaOH and dH2O on ice until use; carry out procedures in hood when possible

APPENDIX C

PROTOCOL FOR SEM IMAGE ANALSYS OF COLLAGEN FIBRILS

SEM Image Analysis Protocol

I. Open ImageJ, and the desired unaltered picture

II. Click on the “Straight line selections” in the “ImageJ” window, and use it to measure the length (in pixels) of the scale bar on the image. Note how many micrometers the scale bar represents. NOTE: Images are 1280X960 pixels in their unaltered form.

III. Crop and save the desired area of the picture. NOTE: Locate areas where the layering of fibrils is such that voids can be seen. Otherwise, ImageJ will not be able to correctly assess what area constitutes a void. Also, the areas should be relatively flat. Image sizes 150X150 pixels.

IV. Image > Zoom > In (2 times)

V. Again using the “Straight line selections” option, randomly measure the width of three fibrils in pixels. Be sure to measure the width perpendicular to the direction the fibril is going (rather than horizontally).

VI. Convert the pixel width to micrometers using the information gained from II: # micrometers (scale bar)/# pixels (scale bar) * # pixels (fibril width) = width of fibril in micrometers

VII. Run “Binary” macro on the crop

```
run("Despeckle");
run("Brightness/Contrast...");
run("Enhance Contrast", "saturated=0.5");
run("Apply LUT");
run("Enhance Contrast", "saturated=2 normalize equalize");
setAutoThreshold();
//run("Threshold...");
setAutoThreshold();
setThreshold(105, 255);
run("Convert to Mask");
run("Histogram");
```

VIII. Jot down the total number of pixels. Then, press “list” and note the number of black pixels (value 0).

IX. Input the total number of pixels (T) and the number of black pixels (B) into the equation: Fibril Area (FA)= B/T (with EXCEL)

X. Run “Skeleton” macro

```
run("Skeletonize");
run("Histogram");
```

XI. Note the total number of pixels. Then, press “list” and note the number of black pixels again.

XII. Input the total number of pixels (T) and the number of black pixels (B) into the equation: Mean Void Area (MVA)= (T-B)/T (with EXCEL)

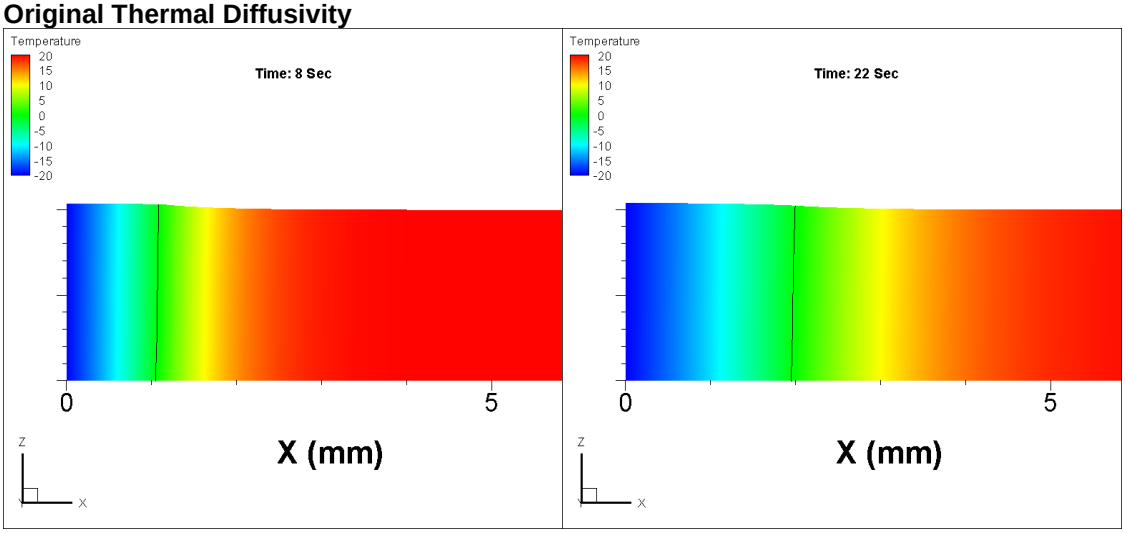
XIII. After acquiring all of the desired crops for a particular section, average the FA and MVA values and find the standard deviation (SD) using the standard EXCEL function and calculate the percent standard deviation (PSD) using the following formula: PSD =100*(1- Avg./(Avg.+ SD))

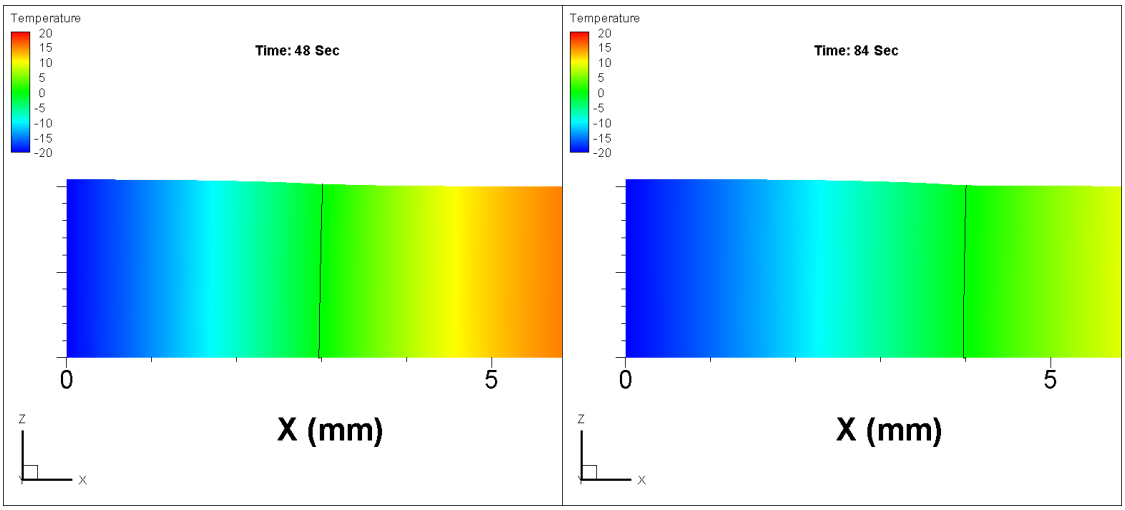
Miller Et al., 2006

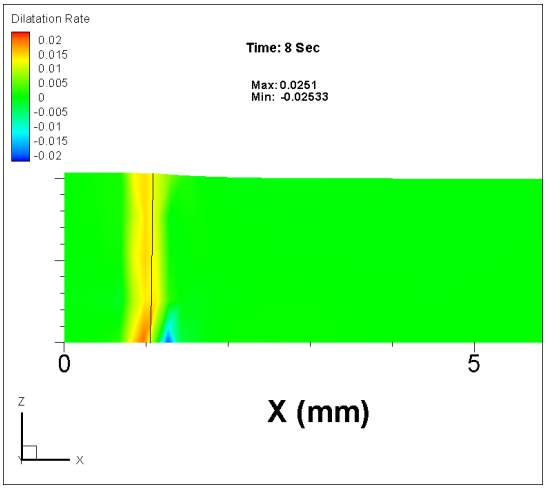
APPENDIX D

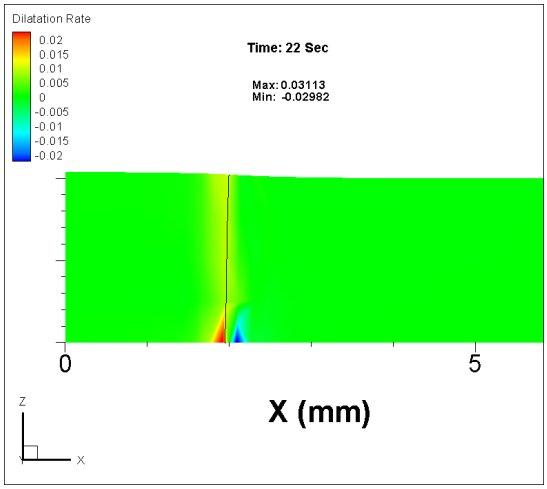
TECHPLOT IMAGES FOR TEMPERATURE, STRAIN RATE, DEFORMATION RATE AND DILITAITON

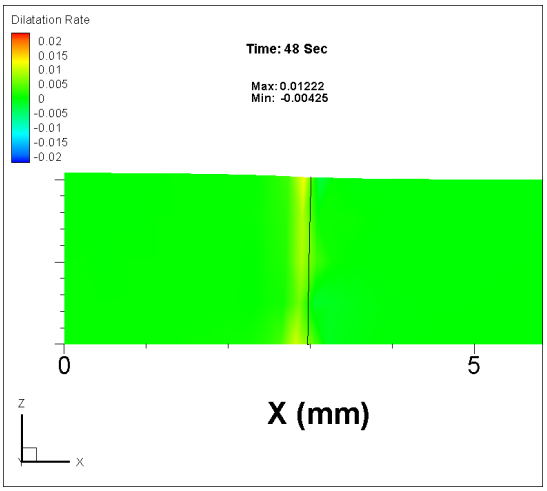
Original Thermal Diffusivity

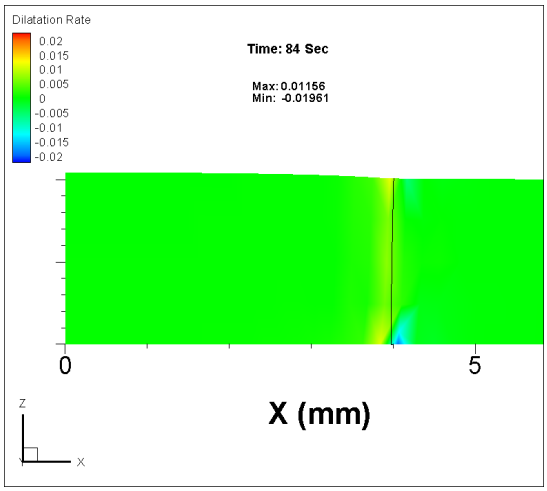




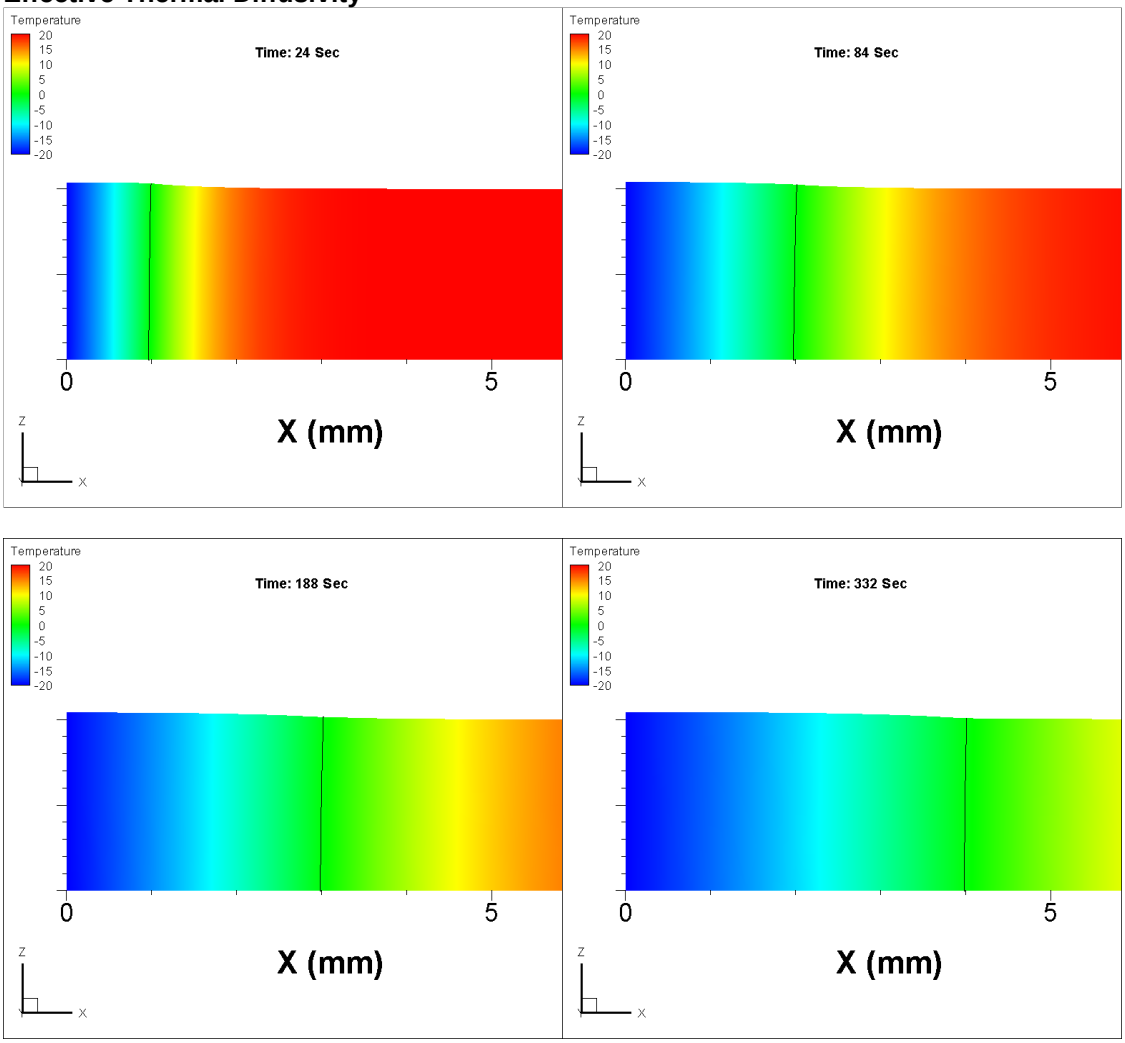

Dilatation Rate
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
Time: 8 Sec
Max: 0.0251
Min: -0.02533
0
5
X (mm)
Z
X

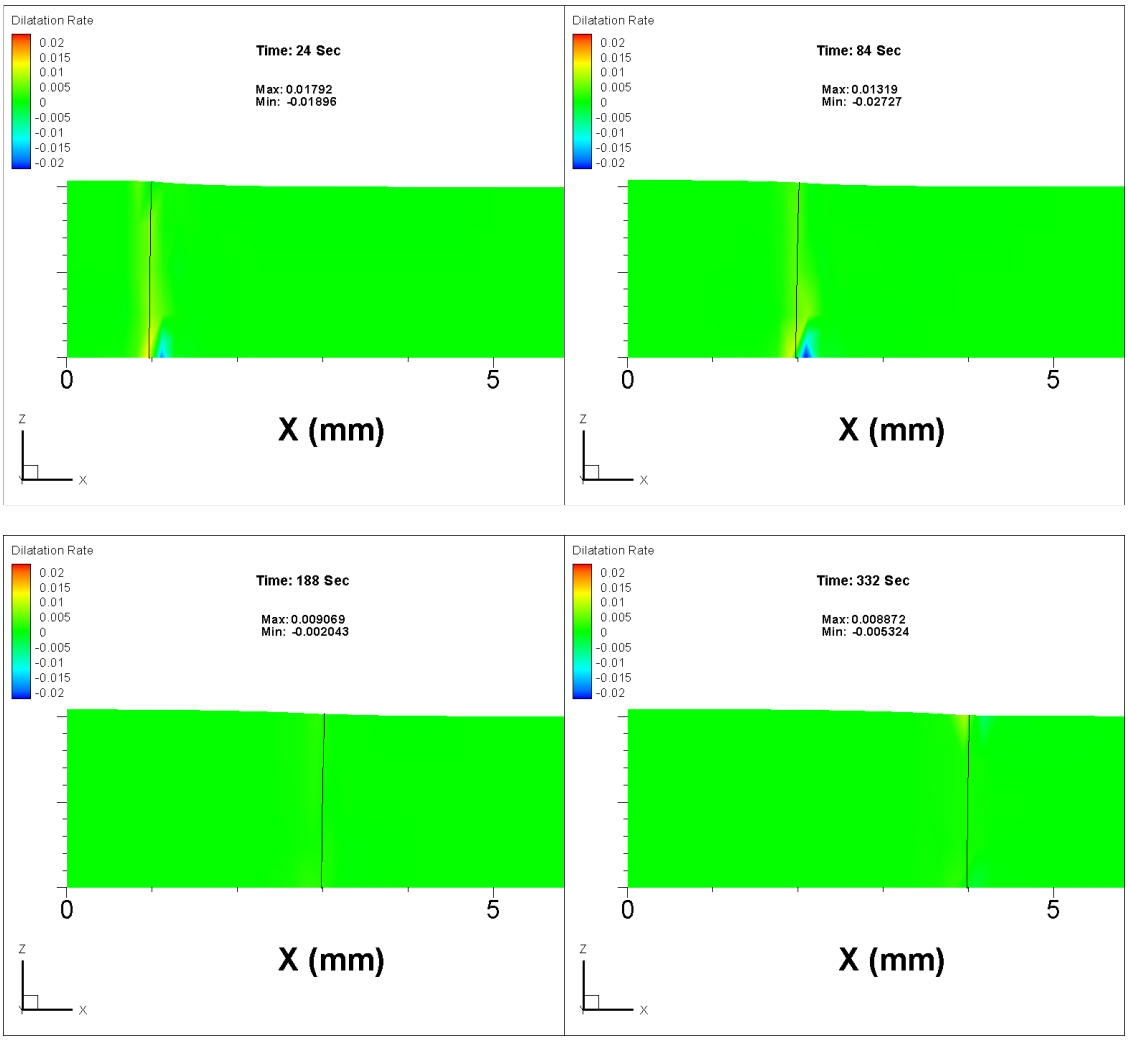

Dilatation Rate
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
Time: 22 Sec
Max: 0.03113
Min: -0.02982
0
5
X (mm)
Z
X


Dilatation Rate
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
Time: 48 Sec
Max: 0.01222
Min: -0.00425
0
5
X (mm)
Z
X


Dilatation Rate
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
Time: 84 Sec
Max: 0.01156
Min: -0.01961
0
5
X (mm)
Z
X

Effective Thermal Diffusivity




Dilatation Rate
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
Time: 24 Sec
Max: 0.01792
Min: -0.01896
0
5
X (mm)
Z
X
Dilatation Rate
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
Time: 84 Sec
Max: 0.01319
Min: -0.02727
0
5
X (mm)
Z
X
Dilatation Rate
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
Time: 188 Sec
Max: 0.009069
Min: -0.002043
0
5
X (mm)
Z
X
Dilatation Rate
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
Time: 332 Sec
Max: 0.008872
Min: -0.005324
0
5
X (mm)
Z
X

APPENDIX E

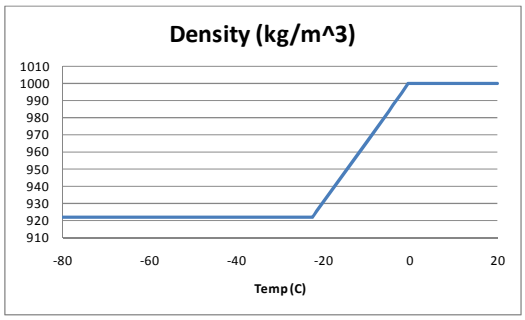
ANSYS SCRIPT AND MATERIAL PROPERTY GRAPHS

```
!With no cheap way to select nodes we go ahead and just select nodes for each run.
!For HCP all working directory references disabled.
/FILNAM, TD_Freezing1,0                                    ! file name
/PREP7
!/CWD,'C:\Documents and Settings\TD_Freeze_600'    ! working directory
SMRT,OFF                                                   !?
/TITLE, VM104, GEL PHASE CHANGE                            ! title
MSHKEY,1                                                   !?
ET,1,SOLID90                                               ! thermal brick element

MPTEMP,1,-80.0,-22.5 ,-22.0 ,  -21.5 ,  -10.0 ,  -05.0 ,
MPTEMP,7,-04.0,-02.0 ,-00.5 ,  00.5 ,   01.0 ,   20.0 ,
MPDATA,DENS,1,1,921.9 ,      921.9 ,  923.7 ,  925.4 ,  966.1 ,  983.8 ,
MPDATA,DENS,1,7,987.3 ,      994.4 ,  999.7 ,  999.7 ,  999.7 ,  999.7 ,
MPDATA,KXX,1,1,       1.996 ,  1.996 ,  1.963 ,  1.930 ,  1.175 ,  0.847 ,
MPDATA,KXX,1,7,       0.781 ,  0.650 ,  0.551 ,  0.551 ,  0.551 ,  0.551 ,
MPDATA,C,1,1,2099.5,2099.5 ,2147.1,2194.7 ,3289.9 ,3766.1 ,
MPDATA,C,1,7,3861.3,4051.8 ,4194.7,4194.7 ,4194.7,4194.7 ,
MPDATA,EX,1,1,8.970E+06 ,8.970E+06,8.766E+06 ,    8.562E+06 ,3.875E+06,1.838E+06 ,
MPDATA,EX,1,7,1.430E+06 ,6.150E+05,3.650E+03 ,    3.650E+03 ,3.650E+03,3.650E+03 ,
MPDATA,ALPX,1,1,-2.296E-04 ,-6.117E-04 ,-6.194E-04 ,-6.233E-04 ,-8.579E-04 ,-9.634E-04 ,
MPDATA,ALPX,1,7,-9.472E-04 ,-6.145E-04 ,7.323E-05 ,7.323E-05 ,7.323E-05 ,0.000E+00 ,
MPDATA,ENTH,1,1,0.000E+00 ,1.113E+08 ,     1.178E+08 ,1.243E+08 ,          2.808E+08
,3.527E+08 ,
MPDATA,ENTH,1,7,3.674E+08 ,3.971E+08 ,4.196E+08 ,4.238E+08 ,4.259E+08 ,
        5.056E+08 ,
MP,NUXY,1,.33

X1=0
X2=.05
Y=.01
Z=.002
K,1,X1,0,0
K,2,X1,0,Z
K,3,X1,Y,Z
K,4,X1,Y,0
K,5,X2,0,0
K,6,X2,0,Z
K,7,X2,Y,Z
K,8,X2,Y,0
L,1,5,
L,2,6,
L,3,7,
L,4,8,
V,1,2,3,4,5,6,7,8
LSEL,S,LOC,X,.025
LESIZE,ALL,,,100,10
ESIZE,0.0005
VMESH,ALL

FINISH
```

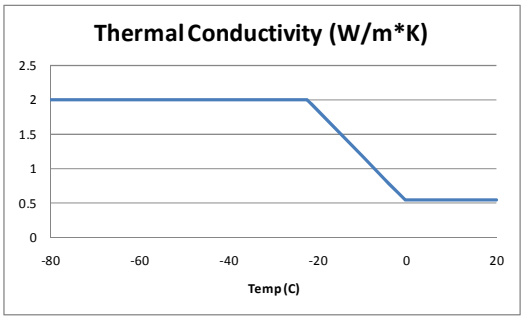
```
/SOLU
ANTYPE,TRANS                    ! Transient Analysis
OUTRES,ALL,ALL                  ! OUTPUT ALL RESULTS
BFUNIF,TEMP,20                  ! INITIAL TEMPERATURE
NSEL,S,LOC,X,0                  ! SELECT ALL NODES AT X=0
D,ALL,TEMP,-20.0                ! ALL NODES AT X=0,TEMP=-20
NSEL,S,LOC,X,0.05               ! SELECT ALL NODES AT X=.05
D,ALL,TEMP, 20                  ! ALL NODES AT X=0.5,TEMP=20
NSEL,ALL                        ! SELECT ALL NODES
KBC,1           ! STEP LOAD
AUTOTS,ON                       ! AUTO TIME STEP ON
DELTIM,.1,.005,1                ! DELTA TIME AND RANGE OF DELTA TIME
TIME,5          ! FINAL TIME
SOLVE
FINISH

!Plots are saved to jpegs at replot with white backgrounds
/REPLOT
/SHOW,CLOSE
/DEVICE,VECTOR,0
/SHOW,JPEG,,0
/GFILE,800,
/CMAP,_TEMPCMAP_,CMP,,SAVE,10
/RGB,INDEX,100,100,100,0
/RGB,INDEX,0,0,0,15
/REPLOT

finish

/Post1
/DSCALE,1,5         !Scale of graphs exaggerate by 5
/CONT,1,10,AUTO     !Contours are divided into 10 sections
/REPLOT
/TITLE, GEL Temp 1,
PLNSOL, TEMP,1, 1               ! PLOT TEMPERATURE
Finish

/PREP7
ETCHG,TTS               ! Switch to structural element, SOLID185
NSEL,S,LOC,X,0                  ! select all nodes at X=0
D,ALL,ux,0                      ! displacement for all select nodes in x-dir is 0

NSEL,S,LOC,z,0                  ! select all nodes at z=0
D,ALL,uz,0                      ! displacement for all select nodes in z-dir is 0
NSEL,S,LOC,z,0                  ! select all nodes at z=0
D,ALL,ux,0                      ! displacement for all select nodes in x-dir is 0
NSEL,S,LOC,z,0                  ! select all nodes at z=0
D,ALL,uy,0                      ! displacement for all select nodes in y-dir is 0

NSEL,S,LOC,Y,0          ! select all nodes at y=0
D,ALL,UY,0              ! Displacement for all select nodes in y-dir is 0
nsel,all
```

```
/solu
OUTRES,ALL,ALL                          ! OUTPUT ALL RESULTS
tref,20                                 ! reference temperature
LDREAD,TEMP,1,nth,,,'TD_Freezing1','rth'  ! Read in temperatures from thermal run
timint,off,all                  ! no time integration -- treat as Steady State
nsubst,2                        ! two substeps zero initial velocity for tansient
kbc,1                           ! step change load
solve
finish

/POST1
/EFACET,1
/DSCALE,1,3                             ! plot scaling factor
/TITLE, GEL Sum 1,                      ! title for plots
PLNSOL, U,SUM, 1,1.0                    ! plots displacement
PLNSOL, S,  1, 1,1.0                    ! plot 1st principle stress
FINISH

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!Begin Loops!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

*DO,Loop,2,120,1                        ! loop  This is set to go for 600s or 10 min
PLoop=Loop-1                            ! previous loop
/FILNAM, TD_Freezing%Loop%,0                    ! file name is based on paramenter "Loop"

/PREP7
ETCHG,STT                               ! Switch elements from structural to thermal
UPGEOM,1,,,TD_Freezing%PLoop%, rst,         ! update the geometry from previoius results

/SOLU
ANTYPE,TRANS                                    ! Analysis type is transient
timint,on,all                                   ! turn time integration on
OUTRES,ALL,ALL                                  ! OUTPUT ALL
LDREAD,TEMP,,,5,2,'TD_Freezing%PLoop%','rth'    ! Initial temperature from previous run
NSEL,S,LOC,X,0                                  ! SELECT ALL NODES AT X=0
D,ALL,TEMP,-20.0                        ! ALL NODES AT X=0,TEMP=-80
NSEL,S,LOC,X,0.05                               ! SELECT ALL NODES AT X=.05
D,ALL,TEMP, 20                                  ! ALL NODES AT X=0.5,TEMP=20
NSEL,ALL
KBC,1                   ! STEP LOAD
AUTOTS,ON                               ! AUTO TIME STEP ON
DELTIM,.1,.005,1                        ! DELTA TIME AND RANGE OF DELTA TIME
TIME,5                          ! FINAL TIME
SOLVE
FINISH

/Post1
/DSCALE,1,3                             ! Scale of plots
PLNSOL, TEMP,1, 1                       ! output temperature plot
Finish

/PREP7
```

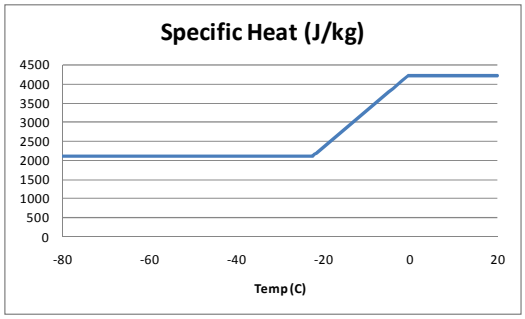
```
!/Clear                                  ! clear database
Resume, TD_Freezing1,RDB,,1              ! load original geometry
ETCHG,TTS                          ! Switch to structural element, SOLID185
NSEL,S,LOC,X,0                           ! select all nodes at X=0
D,ALL,ux,0                               ! displacement for all select nodes in x-dir is 0

NSEL,S,LOC,z,0                           ! select all nodes at z=0
D,ALL,uz,0                               ! displacement for all select nodes in z-dir is 0
NSEL,S,LOC,z,0                           ! select all nodes at z=0
D,ALL,ux,0                               ! displacement for all select nodes in x-dir is 0
NSEL,S,LOC,z,0                           ! select all nodes at z=0
D,ALL,uy,0                               ! displacement for all select nodes in y-dir is 0

NSEL,S,LOC,Y,0                     ! select all nodes at y=0
D,ALL,UY,0                         ! Displacement for all select nodes in y-dir is 0
nsel,all

/solu
tref,20                                  ! reference temperature
LDREAD,REAC,1,nth,,,'TD_Freezing%PLoop%','rst'       ! Apply reactionary loads from mech
run
LDREAD,TEMP,1,nth,,,'TD_Freezing%PLoop%','rth'  ! Read in temperatures from thermal run
timint,off,all                           ! no time integration -- treat as Steady State
nsubst,2                                 ! two substeps to imply zero initial velocity for transient
kbc,1                                    ! step change load
solve
finish

/POST1
/EFACET,1
/DSCALE,1,3                              ! plot scaling factor
/TITLE, VM104, TD_Freezing %Loop%, ! title for plots
/CONTOUR,1,10,0,AUTO
PLNSOL, U,  SUM, 1,1.0                   ! plots displacement
PLNSOL, S,    1, 1,1.0                   ! plot 1st principle stress
PLNSOL, EPEL, 1, 1,1.0                   ! plot 1st principle strains

FINISH

*ENDDO

/SHOW, TERM                              ! all future plots are sent to screen

Finish
```



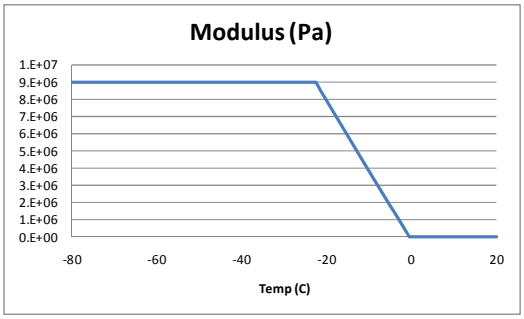
From reference [11]



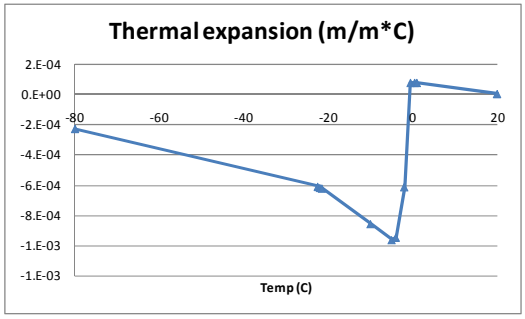
From reference [11]



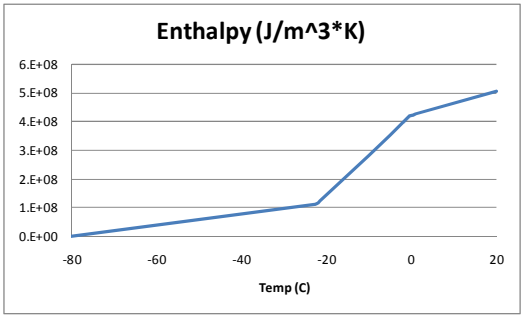
From reference [11]



From reference [13]



From reference [12] modified for this study

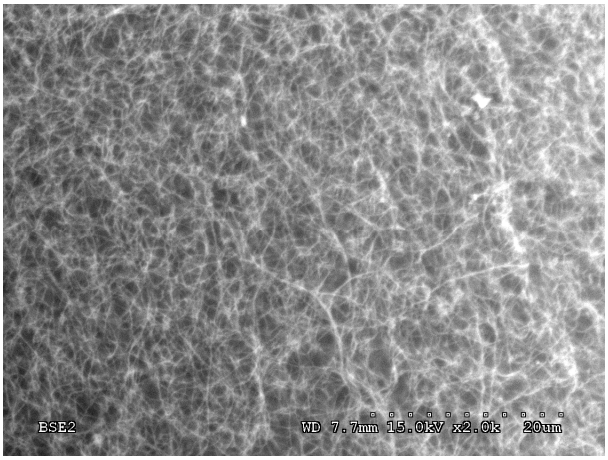


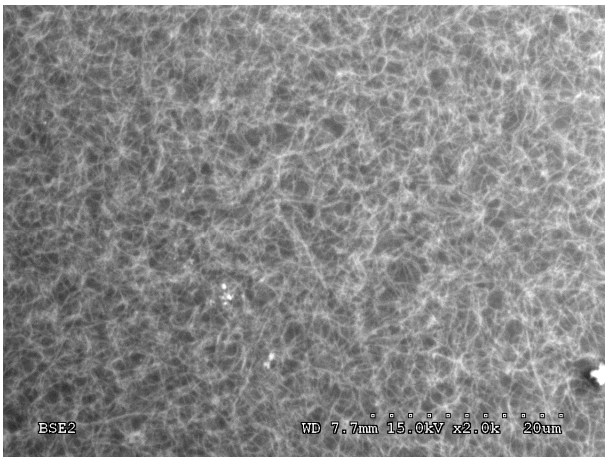
From reference [14]

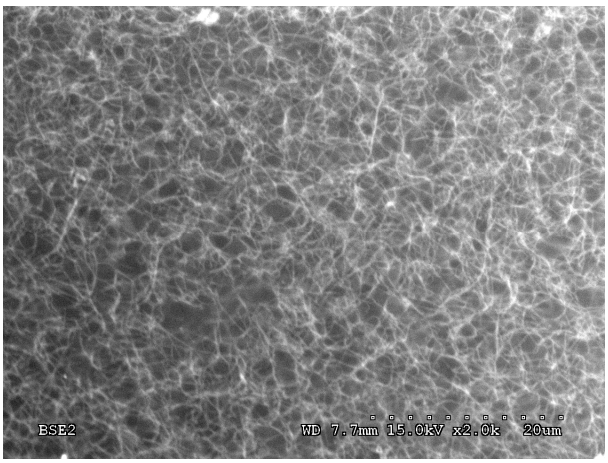
APPENDIX F

ORIGINAL SEM IMAGES OF ENGINEERED TISSUE

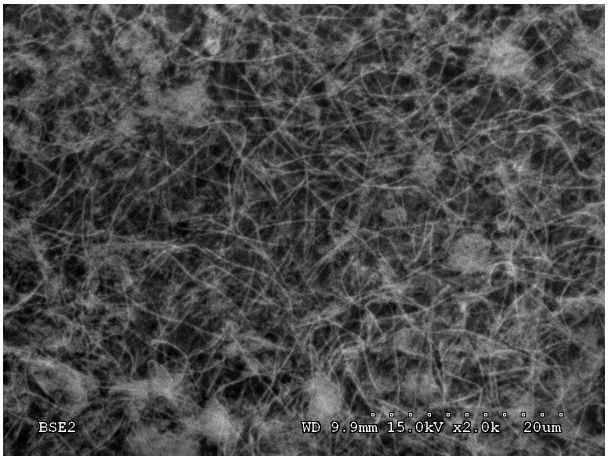
Unfrozen SEM images of 3 mg/ml collagen with nominal cell density

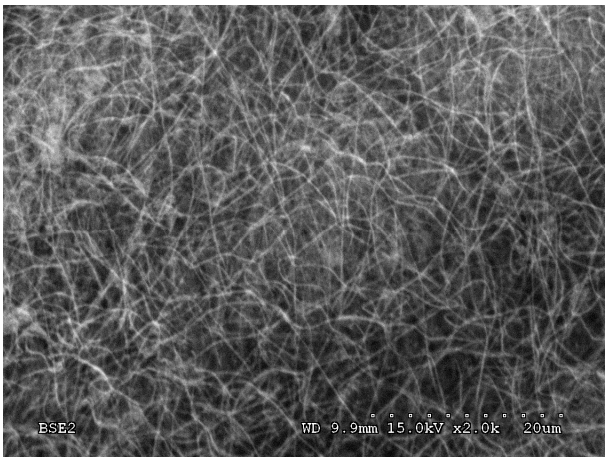


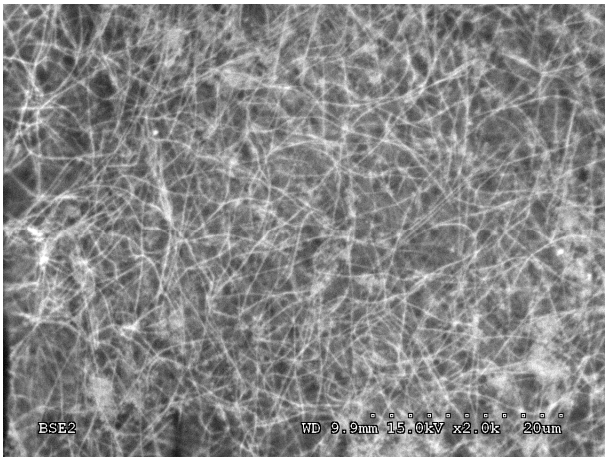




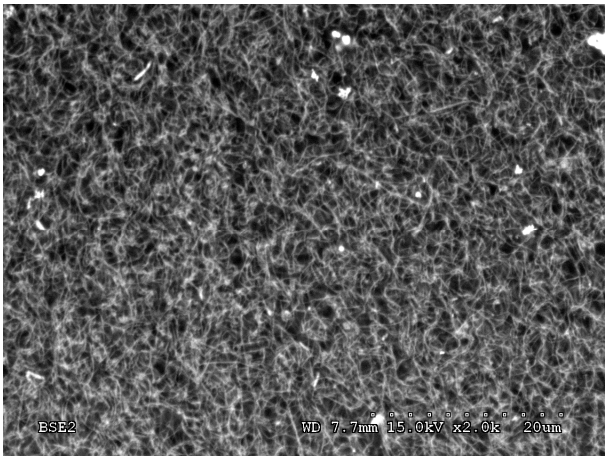
Frozen/thawed SEM images of 3 mg/ml collagen with nominal cell density

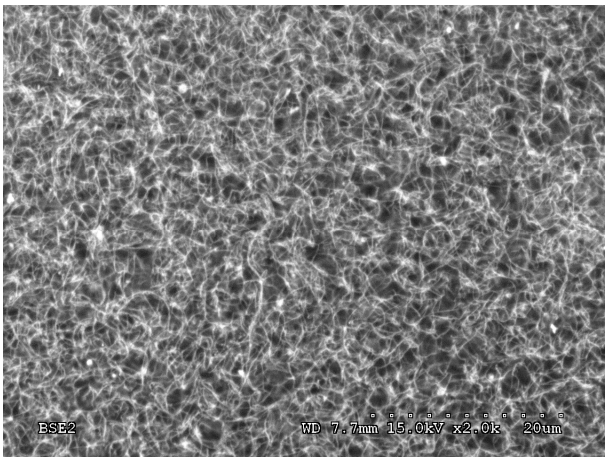


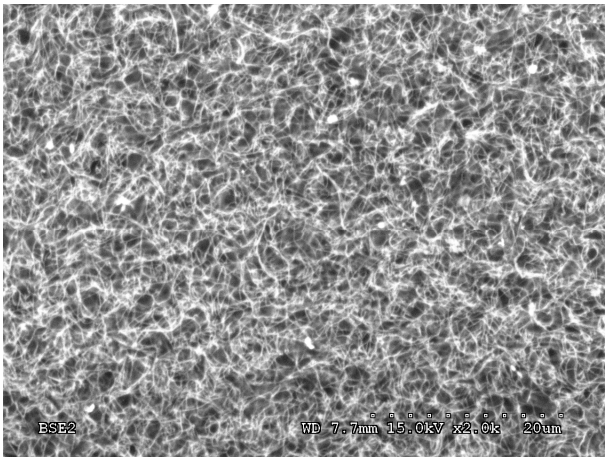




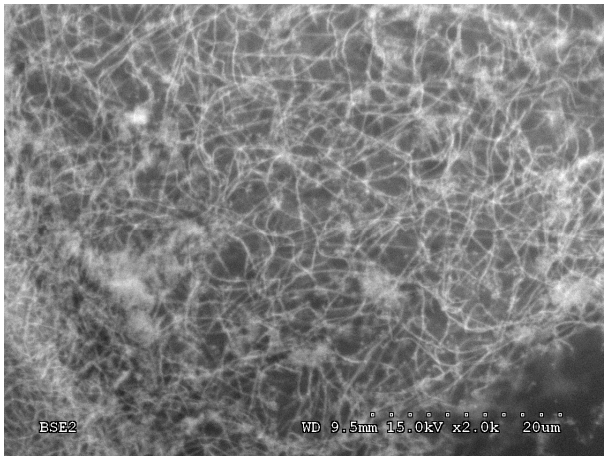
Unfrozen SEM images of 3 mg/ml collagen with high cell density

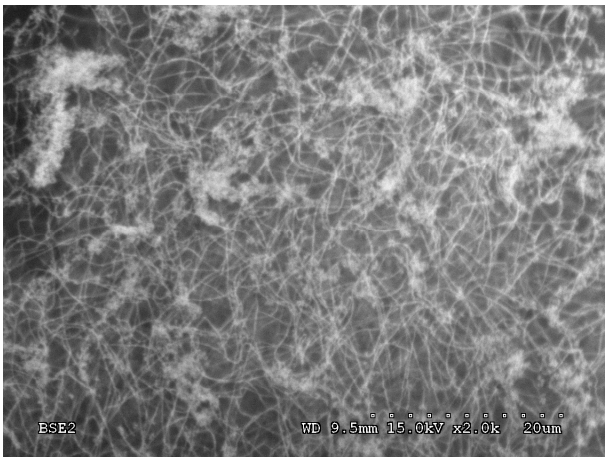


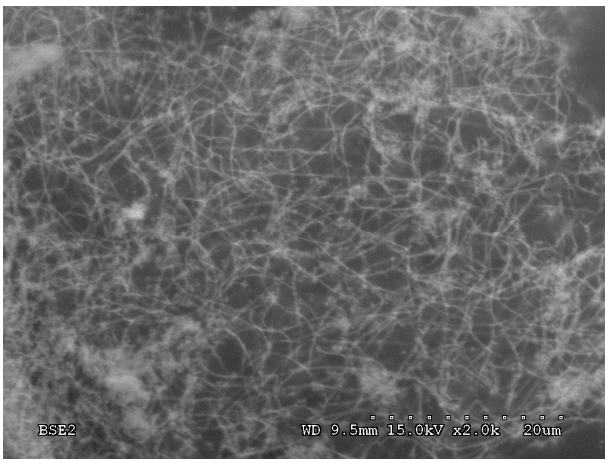




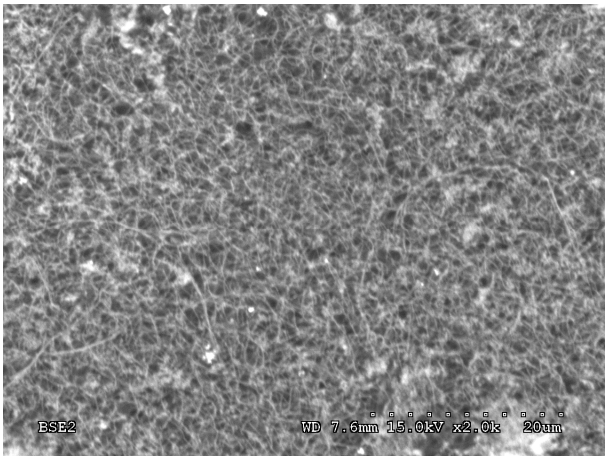
Frozen thawed images of 3 mg/ml collagen with high cell density

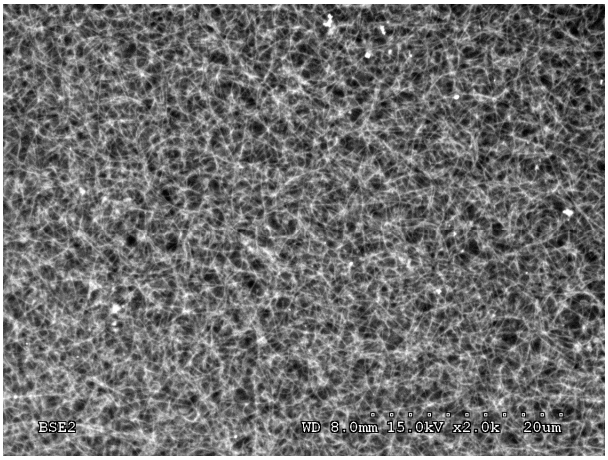


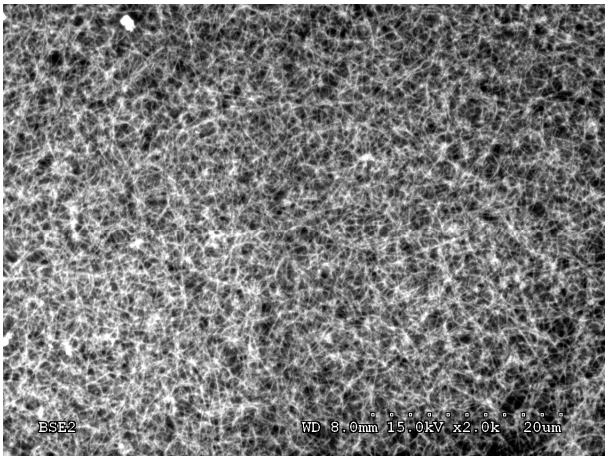




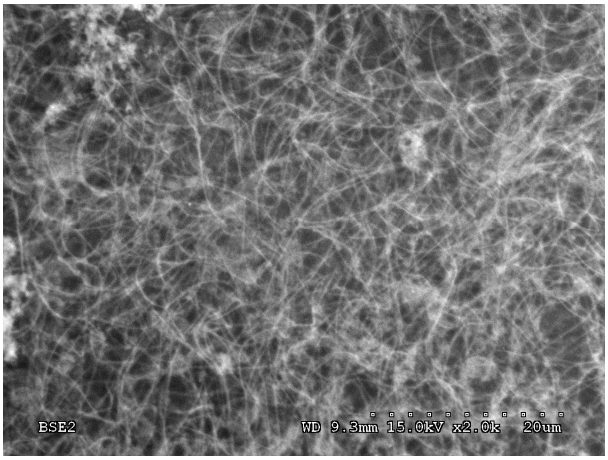
Unfrozen images of 6mg/ml collagen with nominal cell density

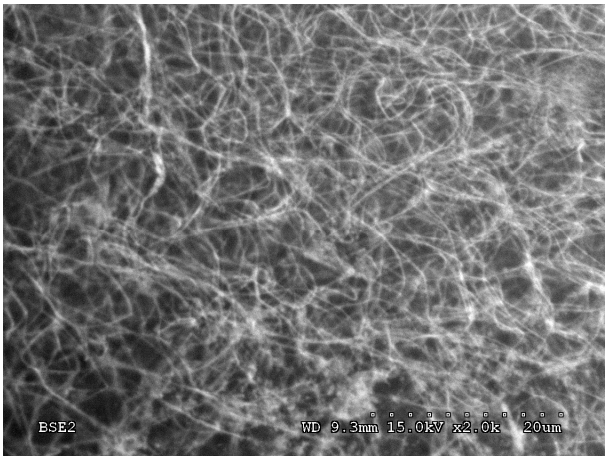


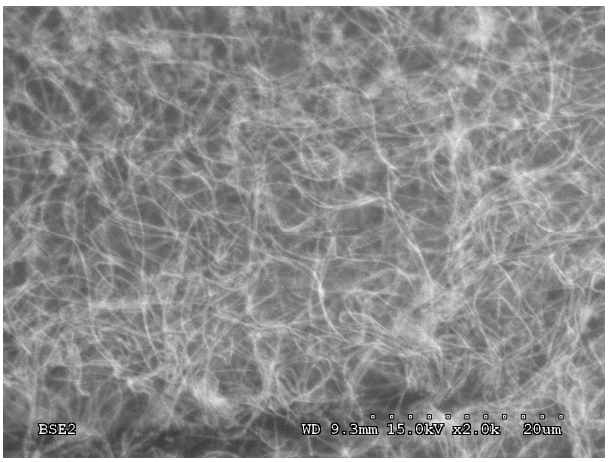




Frozen thawed images of 6mg/ml collagen with nominal cell density







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BIOGRAPHICAL INFORMATION

Tenok DeHoyos is a graduate from the University of Texas at Arlington. His undergraduate work was also in mechanical engineering with interest in thermal design for electronic packages. Tenok has a soft spot in his heart for thermodynamics and would enjoy pursuing his work in that field. Born at the foot of the Rocky Mountains in Utah, Tenok moved at a young age with his family to Texas. It took some time look pass the heat and mosquitoes but once he did he's fallen in love with this great state.