BME 201 Design Project Final Report

Project Name
Periodontal Bone Graft and Bioreactor Design

Client:
NP Biomedical

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Biomedical Engineering 201
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Team Members
Ethan Frohna (Team Leader)
Joe Macksood
Courtney Mohs
Elizabeth Liu
Sarah Edwards

Abstract

This paper describes the brainstorming process, fabrication, and testing performed to create a functional bioreactor that could successfully monitor the temperature of the solution, create a sample holder capable of holding six bone grafts, and create a bone graft with similar mechanical properties to native periodontal bone. This system had to be capable of maintaining synthetic bone graft samples for a period of two weeks. To accomplish this, multiple circuit schematics and sample holders were brainstormed before the most effective designs were constructed. Once the circuit was created, it was tested to ensure the thermistor could accurately read several different water temperatures. These tests concluded that the thermistor was able to accurately define the temperature of the water to within an average of 2 °C for three different temperature samples. The sample holder was unable to be fabricated, but it was fully constructed in SolidWorks to aid in visualization. Multiple biomaterials were also considered and evaluated in a design matrix before choosing calcium sulfate. Overall, this project resulted in an effective bioreactor system and a good bone substitute in calcium sulfate. Both the Young's modulus and ultimate strength of the material were maintained throughout the testing period as all differences in these properties between the weeks were not found to be statistically significant.

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Introduction

Motivation

Skeletal defects resulting from trauma or other diseases remain a major clinical problem world-wide. Over 500,000 bone graft procedures occur in the United States alone each year. Specifically, one of the most common applications for bone grafts is for periodontal procedures [1]. Some of these applications include the correction of bone cavities, segmental bone defects, alveolar ridge preservation, and benign bone lesions [2]. The demand for synthetic bone grafts and biomaterials stems from a need to perform specific surgeries on a variety of different patient injuries. By creating a synthetic bone graft material that is relatively inexpensive, easy to fabricate, and capable of promoting natural bone growth, orthopedic surgeons can repair complex fractures using simple biomaterials. There is a wide variety of synthetic biomaterials such as PMMA, calcium phosphate, and calcium sulfate. However, each material has its advantages and disadvantages and can impact the needs and rehabilitation of the injured patient.

Problem Statement

The client, NP Biomedical, has requested the fabrication of a bone graft that retains the mechanical properties of natural bone *in vivo* for two weeks. It must fill a bone gap that is 20 mm deep by 16 mm diameter. The budget is $50 per team to make the synthetic bone graft as well as a bioreactor device to maintain physiological conditions.

Background

Anatomy of Bone

Bone is a rigid but flexible intercellular material that is composed mostly of collagen and calcium phosphate. There are three types of bone tissue: compact tissue, which is the hard outer layer of bones, cancellous tissue, which consists of the sponge-like tissue found on the inside of bones, and subchondral tissue which can be found along the edges of bones. The main functions of bone include: providing shape and support for the body, offering protection for some organs, and providing a medium for the development and storage of red blood cells in the marrow [3].

Human Body Conditions

There are several variables that contribute to normal human body conditions. These include temperature, pH, and salinity. Normal internal body temperature varies based on gender, recent activity, food, and fluid consumption. However, typical temperatures range between 36.5 degrees Celsius to 37.2 degrees Celsius [4]. The range for normal blood pH is tightly constrained to be between 7.35 and 7.45 [5]. Salt is also essential to the human body, but too much can raise blood

pressure and lead to heart disease. The amount of salt in the body is controlled by the brain. When concentrations get too high, the brain sends out a signal to make the person thirsty. Excess salt can then be excreted through urine.The kidneys also work to either excrete or maintain water to balance salt levels [6]. On average, salt makes up approximately 0.4% of the body's weight. This corresponds to 9 grams of salt per 991 grams of water [7].

Causes for Periodontal Bone Grafts

Periodontitis is the main cause for periodontal bone grafts. This disease is defined as a type of inflammation that destroys the supportive alveolar bone and periodontal ligament. This inflammation is caused when plaque builds up on the teeth. This plaque causes periodontal bacteria normally found in the human mouth to increase dramatically, This increase in bacteria initiates an immune response that can lead to devastating inflammation. This inflammation can eventually cause damage to the jaw bone or teeth [8]. Various procedures have been used to eliminate the anatomical defects caused by periodontitis including open flap debridement (OFD), natural or synthetic filling materials (bone grafts), and guided tissue regeneration (GTR) [9]. Historically, synthetic bone graft materials have been used with great success.

Bone Graft Materials

There are three major synthetic biomaterials that are currently used to treat periodontal bone defects: calcium sulfate, calcium sulfate, and PMMA. Each of these biomaterials can be useful as bone substitutes during surgery and bone repair.

Calcium sulfate is a kind of osteoconductive and biodegradable ceramic that has a reabsorption rate of 30-60 days [10] and is well suited to fill small bone defects such as tooth cavities [11]. Its bioabsorbable [12] and osteoconductive properties [13] promote fibroblast migration [14] and help to prevent inflammation [15]. Calcium sulfate can also limit the activity of bacteria in the affected zone by producing an acidic environment when undergoing dissolution [2]. The beta part can be prepared by using drying dishes in an open-air oven heating the calcium sulfate to 150 ºC, 200 ºC, and 240 ºC consecutively [16]. This material has a Young's modulus of 1.45 GPa [17] and peak stress levels of up to 4 GPa [18].

Calcium phosphate is a type of ceramic that is composed of calcium hydroxyapatites. This property gives this material a chemical composition similar to the mineral phase of calcified tissue. It is also bioabsorbable with excellent osteoconductivity. It is primarily used in maxillo-facial surgeries because of its relatively low mechanical strength [19] and is a relatively low-cost option for bone grafts [20]. Hydroxyapatite (HA) is a calcium phosphate that has been extensively used for synthetic bone replacement. HA is biodegradable and very stable compared to other calcium phosphate materials in the pH range of 4.2-8.[21]. However, HA is not

resorbable [22]. This material has a compressive strength of 14-24 MPa [23] and a Young's modulus of 375 MPa [24].

Polymethyl methacrylate (PMMA) is an acrylic polymer that is formed by mixing two sterile components. These consist of a liquid MMA monomer and a powdered MMA-styrene copolymer. As they are mixed, the liquid monomer polymerizes around the pre-polymerized powder particles to form the hardened PMMA. Heat is also generated in the process as it is an exothermic reaction. Usually, the PMMA bone cement is combined with several types of antibiotics to aid in reducing rejection by the patient. The average cost of using this type of cement in surgery is about $600 [25]. One of the main disadvantages of this type of bone cement in joint replacement is cement fragmentation and foreign body reaction to wear debris. This can often result in prosthetic loosening [19]. The Young's Modulus for this material is 2855 MPa [26].

Another important thing to keep in mind when developing this design are FDA codes and regulations. When dealing with biomaterials for use in bone grafts is The Food and Drug Administration's (FDA) guidelines for the verification and validation of the bone grafts materials. The specific stipulations can be found in their "Use of International Standard ISO 10993-1, 'Biological evaluation of medical devices = Part 1: Evaluation and testing within a risk management process'" document [27]. The bone graft created in this project would be classified as a Class II medical device. Since bone grafts are injected, they can pose a moderate risk of illness and injury to the patients such as inflammation, infection, and rejection of the bone graft. The bioreactor would be classified as a Class I medical device according to FDA regulations because it poses little to no harm to the patients [28].

Client

NP Biomedical is a biomedical company run by Dr. Puccinelli and Dr. Nimunkar.

Product Design Specifications

The client has asked the team to construct a bioreactor that is capable of maintaining biological human body conditions in regard to temperature, pH, and salinity for a total of two weeks. It must also contain a sample holder of some sort that is capable of housing 6 synthetic bone samples. The bone samples must be made from a material comparable to native periodontal bone and must be 20mm long x 16mm in diameter. They must maintain mechanical properties similar to native bone for the total duration of two weeks. The entire PDS can be found in Appendix A.

Competing Products

There are several competing bone graft materials that are currently available. One is called Pro-Dense and functions as an injectable regenerative graft composed of calcium phosphate and

calcium sulfate in a 75% to 25% ratio respectively. Since both of these materials are bioabsorbable, it can be reabsorbed into the body after a period of time. Overall, clinical success rates have remained high with a to 86.5% success rate in the femoral head region of the hip joint. Within that same study, 78.4% of the patients showed no hip collapse [28]. Another product on the market is Vitoss: Synthetic Bone Graft. This material is made by Stryker and features an interconnected, ultra-porous structure that resembles human cancellous bone. This product comes in one of three formulations: a foam pack, a foam strip, or morsels and blocks. It is composed of calcium phosphate [29].

Preliminary Designs

Electronics Circuit Design

A voltage divider connected to a non-inverting operational amplifier was used for the thermistor circuit design. Five Volts are directed to the voltage divider containing the thermistor. That voltage is then amplified by the op amp which will display a voltage between 0 and 5 Volts which are the voltages read by the Arduino. See Appendix B for Figure 1, a circuit diagram detailing the design along with the equations used to calculate output voltage and gain. Also see Appendix C for block diagrams relating to this design.

Sample Holder Designs

Modular Disk Design

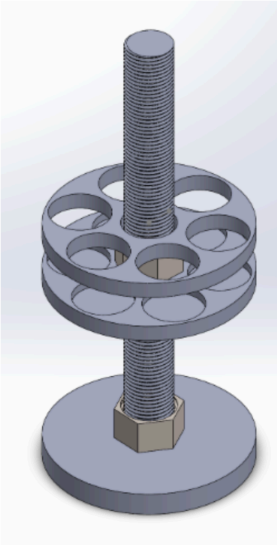


Figure 1. *The Modular Disk Design has three disks that are centered around a threaded rod. The modular design allows the holder to be capable of holding more bone graft samples if needed for future experiments.*

The “Modular Disk Design” consists of two disks that work to support the bone grafts while a third disk serves as a stabilizing base for the entire design. The top disk contains six holes that are arranged in a radial pattern around the disk that go all the way through the material. The middle disk also has six identical holes, but the holes only go halfway through the material. These divots serve to support the bone graft samples after they are slid through the holes in the top disk. The two disks are separated the correct distance of approximately 20mm by placing a threaded hex nut in between both disks. There is also a hex nut underneath the second disk to support it and prevent it from sliding down towards the base. The hex nuts serve as a way to constrain the disks in addition to weighing down the sample holder in the bioreactor solution. This component helps to ensure the sample holder remains stable and the bone graft samples are protected. There is also a threaded rod running up through the center of the entire device that the disks can be threaded onto through a hole in their center. This rod can also function as a handle when pulling the sample holder out of the bioreactor. This design can easily be manufactured with the use of the laser cutter and acrylic.

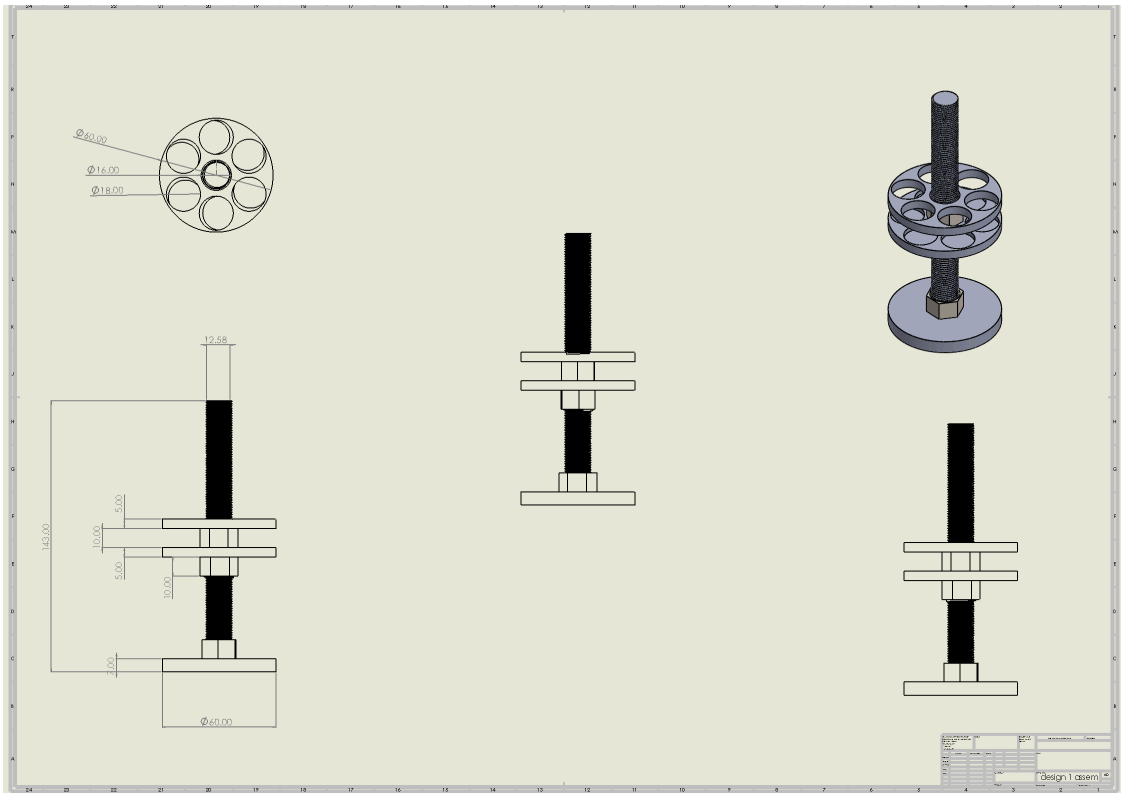


Figure 2. *SolidWorks drawing of the Modular Disk Design with dimensions*

“Plunger” Design

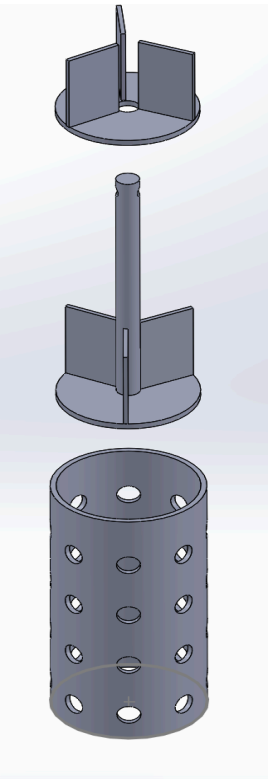


Figure 3. *An exploded view of the three piece system. All of the parts fit together concentrically allowing them to be swapped in and out readily without losing the samples.*

The Plunger design consists of three separate parts that fit together to create one sample holder system. The first part is the shell which fits around the entire apparatus. This hole filled shell allows fluid to flow through the system without letting the sample pieces escape from their holding chamber. This shell is then slid over the remaining two parts, the bottom divider and the modular addition. The bottom divider consists of a plate with a rod in the middle. Attached to this rod are three dividers. These separate the volume into three sections allowing three samples to be placed on one level. The bottom divider’s rod reaches up to the top of the cap, allowing the user to pull out the entire sample holder apparatus. Lastly, the additional module is a plate with the three dividers spaced around a hold in the base. This piece can be slid over the rod on the bottom divider, allowing for more samples to be added. In total, this system is able to hold nine samples among three different levels. The entire weight of the system holds it in place vertically with the shell being concentric and flush with the jar entrance to stabilize the system horizontally. These can all be made with the laser cutter and plastic that can be purchased in the makerspace. The dimensions for this design can be found in Appendix D.

Dreidel Design

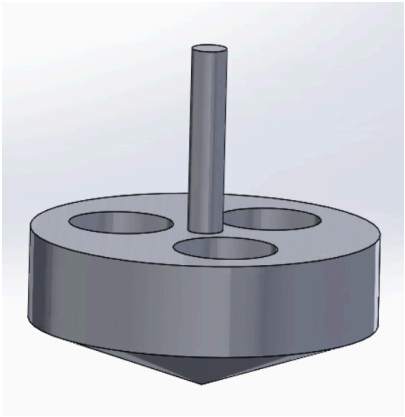


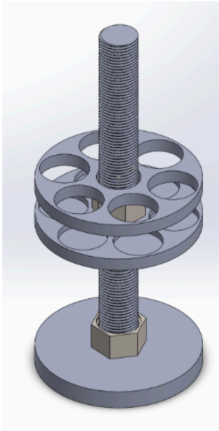
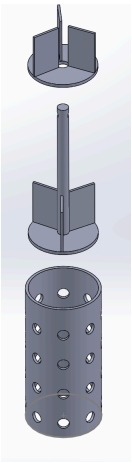
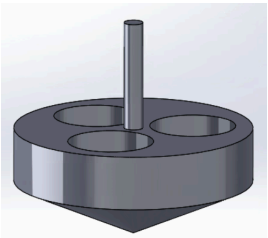
Figure 4. *A lateral view of the Dreidel Design. This design is meant to float on top of the bioreactor solution.*

The Dreidel design consists of a simple cylindrical holder with a cone and rod feature that increases stability and handling. The idea of this design is to take advantage of some of the properties of the plastic and create a floating piece that holds the materials. The design has a main cylindrical shape with indents that holds up to three samples. The bottom is cone-shaped which helps increase the stability of the floating holder. This allows the center of mass to be concentrated in the middle of the holder which prevents it from tipping over. Another feature is a thin rod that is attached in the middle of the piece. This rod serves as a handle and allows the user to take out the holder from the bioreactor. A potential downside to this design is that it can only hold three samples and it restricts how much contact the samples have with the solution. Dimensions can be found in Appendix E.

Preliminary Design Evaluation

Sample Holder Design Matrix

Table 1. *The design matrix used to determine the final sample holder design. The Modular Disk Design was found to be the most effective design.*

	Modular Disk Design		Plunger Design		Dreidel Design	
						
Ease of Manufacturing (30)	5/5	30	1/5	6	1/5	6
Effectiveness (25)	4/5	20	4/5	20	2/5	10
Ease of Use (20)	5/5	20	4/5	16	2/5	8
Durability (10)	3/5	6	3/5	6	2/5	4
Cost (10)	3/5	6	2/5	4	3/5	6
Safety (5)	5/5	5	5/5	5	5/5	5
Total (100)	**87**		**57**		**39**	

The three highest weighted categories were ease of manufacturing ease of use and effectiveness. Ease of manufacturing is important because the only available tool to create the final sample holder was the laser cutter in the Makerspace. Effectiveness was weighted second because the device must have some way of either latching onto the bottom of the bioreactor, or be sufficiently heavy to ensure it can stay completely submerged while testing. It also must remain upright and have no risk of tipping over. The Ease of Use category was also weighted heavily because in order to retrieve the sample holder from the bioreactor, one must pass their hand through the narrow opening in the corning jar and reach in to get the samples out. The final design for the bone graft sample holder must have some sort of handle for the user to be able to reach and pull out the bone graft samples without disturbing the jar and its contents. See Appendix F for detailed descriptions of all design criteria.

Proposed Final Design

As a result of the design matrix scores, the Modular Disk Design was chosen as the final sample holder design. This design scored well in Ease of Manufacturing because it can be easily manufactured with the available tools, namely the laser cutter in the Makerspace. This design also scored well in both the Ease of Use and Effectiveness categories because it offers a simple way to extract the device from the bioreactor (through use of the rod-handle) and allows for the total exposure of the bone grafts to the surrounding bioreactor solution. It is also adaptable to layer multiple disks on top of each other to test more samples, or to hold the samples in place in case they start to move around. By utilizing a more open design, the bone grafts will be unobstructed from the effects of the bioreactor solution. This will allow the team to get more accurate results in terms of how the bone grafts are mechanically tested at the end of the testing period.

Biomaterial Evaluation

Three biomaterials were considered for use as a synthetic bone graft for this project. These materials included: calcium sulfate, calcium phosphate, and polymethyl methacrylate (PMMA). See Appendix G for full chemical reactions of each.

Biomaterial Design Matrix

Table 2. *The design matrix used to determine the final bone graft material. Calcium Sulfate was found to be the best biomaterial.*

	Calcium Sulfate $CaSO_4$		**Calcium Phosphate $Ca_3(PO_4)_2$**		**Polymethyl methacrylate (PMMA)**	
Ease of Fabrication (25)	4/5	20	2/5	10	1/5	5
Durability (20)	5/5	20	3/5	12	4/5	16
Likeness to Native Bone (20)	4/5	16	4/5	16	4/5	16
Cost (15)	4/5	12	4/5	12	3/5	9
Biocompatibility (10)	5/5	10	4/5	8	5/5	10
Safety (10)	4/5	8	4/5	8	4/5	8
Total (100)	**86**		**66**		**64**	

To determine the best design for the bone graft material, the team evaluated the three materials shown above on predetermined criteria considered most significant in the design. See Appendix H for more detailed descriptions. Each criterion was weighted accordingly and the final scores were calculated to determine the most effective final design for the bone graft material. The top three weighted categories are: Ease of Manufacturing, Durability, and Likeness to Native Bone. Ease of Manufacturing is ranked highest because of time constraints and tools available to fabricate the bone grafts. Next, is Durability because the bone graft needs to withstand the forces native periodontal bone is commonly subjected to. This category was evaluated by looking at the material's stress values and Young's modulus. Likeness to Native Bone is also important because the materials have to behave similar to native bone in the chemical sense in order to prevent infection or rejection by the patient.

Proposed Final Bone Graft Material

As a result of the design matrix scores, calcium sulfate, specifically calcium sulfate dihydrate was chosen as the best bone graft material. This material scored well in cost as it was the least expensive of all three materials ($26 compared to $35 for calcium phosphate and $58 for PMMA). It also scored well in Ease of Fabrication because it can be purchased in a form that only requires water and relatively mild heating in order to create a workable, bone-like material. This makes the samples very easy to fabricate. Finally, this material scored well in the Biocompatibility category because it is osteoconductive, is bioabsorbable, and rarely causes infection.

Fabrication/Development

Materials

The materials for this project were going to be purchased from the UW Makerspace, Grainger, and VWR International. The material for the synthetic bone graft was chosen as calcium sulfate dihydrate. The materials for the bone graft sample holder included acrylic plastic to create the disks, a nylon threaded rod to support the disks, and multiple hex nuts to secure the disks in place. Acrylic plastic was chosen due to it being readily available from the makerspace. In addition, it is cost effective and can be cut using a laser cutter. It can also easily withstand physiological conditions since it has a melting point of 160 degrees C [30]. With a melting temperature this high, the acyclic can withstand the standards of steam sterilization for bioreactors so that it can be reused again [31]. Also, if steam sterilization is not able to be used, acryl can easily be sterilized with chlorine solution without damaging its chemical properties [32].This is well below the typical physiological conditions expected in the bioreactor mentioned in the Introduction. Acrylic plastic is also insoluble in water so it will hold its form when placed in water [33]. The nylon rod was chosen as the supporting component. Like acrylic material, it is

very heat resistant and does not dissolve or lose its structural integrity in water. Also, it is a standard material used in many bioreactors[34]. Glass is also another commonly used material as it is heat resistant and easy to sterilize, making it one of the most common materials used in bioreactors[35]. The corning jar given fits this profile for the containment device. Lastly nylon hex nuts were chosen as they can be used in the threaded nylon rod to secure the plates together. The hex nuts were chosen so that they would not corrode as easily in a saltwater based solution. The materials for the electronic circuit consisted of various components from the team's electronics kits. These materials included an Arduino Uno microcontroller (to write the temperature recording code to), a thermistor (to convert voltage values to temperature values), multiple resistors, an operational amplifier, a breadboard (to construct a working electronic circuit), and a wifi chip (to send data collected by the thermistor to a google spreadsheet for monitoring by the team). There were also several pieces of equipment given to the team by the BME Department in order to construct the heating element of the bioreactor. These included: a beefcake relay, corning jar, heat resistant pad, heating element, corning jar lid, LED display, and wiring. Due to unforeseen circumstances with the COVID-19 pandemic, none of these materials were actually purchased. See Appendix I for a complete list of materials used.

Methods

Sample Holder Fabrication Methods

The main tool that would have been used to fabricate the Modular Disk Design is the laser cutter. The tools available for use in the fabrication were limited due to TEAM lab damage. Also, the fabrication of the sample holder was not able to actually be completed. The following fabrication method details how the team would have constructed this design if it had been possible. First, three 60 mm disks would have been cut from ⅛ inch thick acrylic sheeting. (Further testing of acrylic can be conducted according to FDA guidelines [36]). Once complete, six holes would have been cut in a radial pattern 5 mm from the edge of the disk. Next, another six holes could have been cut in another one of the large disks, but only to a depth of 1.5mm instead of all the way through. Lastly, one hole would have been cut in the center of the final remaining disk to a depth of 1.85mm. Then a 16mm diameter hole could be cut all the wall through in the center of the other two disks. The two disks would have then been placed on the nylon rod with hex bolts separating them. The third and final disk with the 1.85 mm deep hole would have been placed on the bottom of the rod. Once all three disks were placed on the rod, the hex nuts could be adjusted to obtain the proper spacing. A more detailed version of this fabrication protocol can be found in Appendix J.

Biomaterial synthesis

Below is a set of instructions for how the calcium sulfate would have been made to create the synthetic bone grafts for this project. The detailed step by step version and value calculations can be seen in Appendix K and Appendix L respectively.

First, put on gloves and retrieve a 50mL and 100mL beaker. Next, fill the 50mL beaker with diH2O. Transfer 28.147mL of diH_2O to 100mL beaker. Take calcium sulfate powder and measure out 42g before placing into 100mL beaker. Allow mixture to still in 100mL beaker with spatula for 3 min, then rest for 2 min. Then, pour the mixture into 9 molds. Place molds onto a vibrating table (6000rpm) and let them sit for 5 min. Then, remove molds and place in a 250mL beaker before putting onto a 90 degree Celsius hot plate. Let them sit for 20 min on plate. If they are not dry by the end of 20 min, let dry in 5 min intervals, making sure to check molds each time until dry [37]. The molds can then be removed from the hot plate and prepared in the biological safety cabinet (BSC), running through the correct starting protocol. Obtain a plastic plate and spray with 70% ethanol and also wipe down the sides of plastic molds with 70% ethanol. Remove plastic from molds and place onto plastic plates. Put the plate into BSC, close the sash and run the UV light for 15 min. Finally, remove molds from BSC and place them into desired areas. Repeat this process again to make a total of 12 molds. Three extra are included to compensate for faulty creation of molds.

Final Design

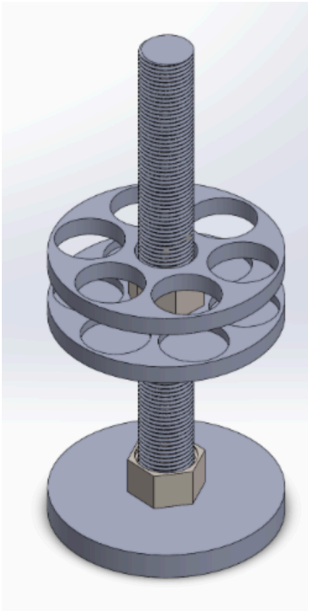


Figure 5. *Final Sample Holder Design (unable to be physically fabricated)*

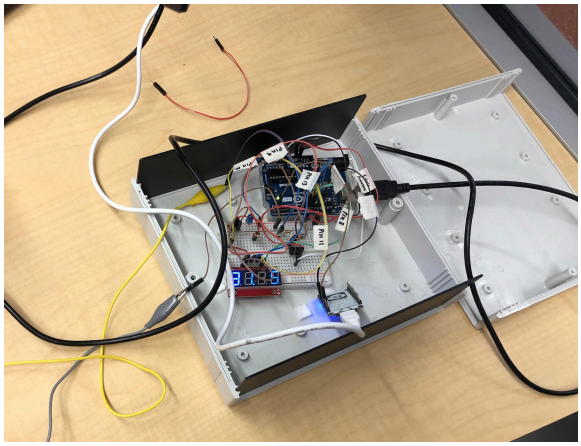


Figure 6. *Final Electronic Circuit and Thermistor Setup*

The final design was not able to be constructed as a result of the COVID-19 pandemic. Had the team been able to complete fabrication, the Modular Disk Design would have been constructed for the sample holder as shown above. Also, the final electronic setup was not able to be completed. Pictured above is a picture of the circuit and seven-segment display before further fabrication was halted. The breadboard, connector wires, seven-segment LED display, wifi chip, and thermistor are visible in the construction shown above.

Testing

Multiple tests were performed on parts of the overall design to make sure that it was compliant with the standards that were set forth by the client and detailed in the PDS.

Thermistor testing

In order to make sure that the samples are kept within the human range, the thermistor had to be calibrated to ensure it was able to accurately read real-world temperature values. A NTCLE413 10K 1 % B3435 K thermistor was selected as it can range between 10k ohms to 5k ohms in resistance, corresponding to 25 °C and 45 °C respectively. These are within the core temperature of the human body (36.1-37.2 °C [38]). The datasheet for temperature and resistance values can be found in Appendix M. To test the accuracy of the thermistor, it was placed in water baths of three different temperatures while the Arduino code was running. After waiting a few seconds for the reading to stabilize, the output temperature reading was recorded. This process was repeated three times for each water bath temperature to obtain an average temperature reading from the thermistor. This value could then be compared to the actual temperature of the water bath in order to assess how accurate the thermistor was. Below is a table of the data collected during testing. The protocol for this test can be found in Appendix N.

Table 3. *The data obtained from the thermistor testing with the actual water bath temperature and average measured temperature with the thermistor.*

Water Bath Temperature (^{0}C)	Expected Voltage (V)	Average Measured Temperature (3 trials)
23	4.25	21.03
27	4.03	26.3
52	2.50	55.01

Electronic Circuit Testing

Preliminary testing was also performed on the circuit to ensure that varying temperatures will result in the predicted voltage output by the equation. This was done to ensure that the temperature data that was received was in fact accurate. When testing the voltage output of the circuit using the Arduino code, the voltage output remained unchanged at 4.99 Volts and would not change with different temperatures. The circuit was rebuilt many times and the resistors were tested but the voltage output did not change. After consulting a TA, the team noticed the thermistor was broken in half. After replacing the thermistor, the results are listed below:

Table 4. *Electronic Testing Results for Thermistor*

Temperature	Estimated Resistance	Estimated Output	Output
25 ^{0}C	10 kΩ	4.255 Volts	4.3 Volts
36 ^{0}C	7 kΩ	3.5 Volts	3.5 Volts

In the range the electronic circuit is designed for, 25 °C to 45°C, the estimated voltage output matches closely with the output displayed by the code.

Microcontroller Code Testing

The microcontroller code had to be tested in order to ensure it was able to work with a wifi chip-data and a seven-segment LED temperature display. To ensure the microcontroller was receiving values expected within the circuit, voltage outputs were inputted manually into the code to verify that the mathematical equation was created correctly. The results are shown in the chart below:

Table 5. *A table showing the testing of the microcontroller code with hard coded values.*

Input vout into code value	Expected Temperature	Code Temperature
4.255	25 ^{0}C	24.68 ^{0}C
2.837	45 ^{0}C	45.06 ^{0}C

The code works correctly, as demonstrated by the circuit testing shown above in the electronic circuit testing section. The code used for this testing can be found in Appendix O. The FDA has

specific guidelines and protocols for testing temperature controls [27]. If further testing could be conducted for this device, FDA guidelines would be followed.

Solidworks Testing

As stated in the Biomaterial Selection section, the team chose to use calcium sulfate for the bone graft material. This material was first tested in SolidWorks to ensure its mechanical properties coincided with a failure in compression as outlined by the client. In order to test the calcium sulfate sample in SolidWorks, three properties of the materials had to be quantified for the simulation to run properly. These included: Poisson's ratio, yield strength, and Young's modulus. Young's modulus for this material is 4.680GPa, the yield strength is 17.7 MPa, and Poisson's ratio is .26 [39]. To simulate the failure of the material while placing it in the jaw bone, the yield strength of the bone must be quantified to make a comparison to the graft's environment. The average ultimate compressive strength of the mandible is 3.9 MPa [40]. Therefore, the maximum force the machine can generate was used in the SolidWorks simulation testing. The force is distributed uniformly over one end of the sample. This was done to simulate the force being applied throughout the entire bone. Since the graft is inside of the jaw, St.Venant's principle can be applied to the jaw as a whole. This results in a uniformly distributed force along the graft. The MTS machine is capable of this by using a solid metal cap that can be assumed to be rigid. The SolidWorks test of a 16x20mm sample piece, fixed at one end with compressive force of 10 kN failed in compressive testing as seen in Appendix P. The lowest Factor of Safety found in the sample is .259069. This shows that the bone graft will fail before the bone structure of the mandible around it does.

MTS Testing

MTS testing data was obtained from pre-recorded data due to the COVID-19 outbreak. Files containing MTS data were given to the team corresponding to the material of choice, calcium sulfate dihydrate. The MTS testing was performed on the bone grafts after being in *vivo* for 0, 1, or 2 weeks respectively. The goal of the test was to determine whether the bone grafts were able to maintain their mechanical properties of Young's modulus and ultimate strength after being in a body-like environment for two weeks. Young's Modulus is the resistance to elastic deformation [65] and the ultimate strength is the maximum tensile, compressive, or shear force a material can sustain [66]. This allowed the team to make conclusions about the potential success or failure of this particular type of bone graft if it were to be used in the real world to fix jaw bone deformities. In particular, the Young's modulus and ultimate strength of the samples were analyzed. The testing protocol used can be found in Appendix Q. This testing was completed with the MTS machine in the BME lab throughout a two week time period. The zero week test served as a control as the bone grafts had not yet been subjected to the *in vivo* environment. The next tests were done after one and two weeks *in vivo* respectively. Three different samples were

used for each test to ensure that the data was consistent between different samples being tested and an average could be obtained. The testing ultimately showed that the bone graft material's ultimate strength and the elastic modulus did not undergo statistically significant changes over the two week test period. Graphs of the stress/strain curves for each week can be seen in Appendix R.

Results

MTS Testing Results

Table 6. *The table below shows the Young's modulus and Ultimate Strength for each bone graft. These values were obtained from analyzing the slope at different points along the stress/strain curves. The run number (ex. Run 1) corresponds to the same bone graft that is tested over time.*

	Run	Young's Modulus (Pa)	Ultimate strength (Pa)
Week 0	2	8.2821e5	5.2197e5
	3	1.5976e6	8.6819e5
Week 1	1	4.5007e4	1.0183e5
	2	6.4997e4	1.5403e4
	3	1.6761e5	6.1053e4
Week 2	1	5.1615e4	1.0251e4
	2	3.4593e4	2.8303e4
	3	4.4858e4	2.6712e4

Statistical Tests

In order to analyze the data collected by the MTS machine, several two-sample t-tests were utilized to determine whether a statistical difference between the average Young's modulus and ultimate strength of week 0 compared to weeks 1 and 2 exists. The null hypothesis was that the mean Young's modulus and ultimate strength of the calcium sulfate samples would be the same between week 0 and week 1, week 0 and week 2, and week 1 and week 2. The alternate hypothesis was that the average Young's modulus and ultimate strength of the calcium sulfate would not be the same between week 0 and week 1, week 0 and week 2, and week 1 and week 2. This type of test was chosen because the team has multiple samples of data to compare and only has knowledge of the sample mean and standard deviations.

P-value and Statistical Significance

For this test, a significance level of 0.05 was used to show 95% confidence. This means that there is a 5% risk of concluding that a difference exists when there is no actual difference. If the p-value is less than this significance level, the differences in the Young's modulus or ultimate strength in week 0 when compared to weeks 1 and 2 can be said to be statistically significant. As a result, the null hypothesis would be rejected. If the p-value is greater than this significance level, the differences between the Young's modulus in week 0 compared to week 1 and 2 are not statistically significant. As a result, the team would choose to fail to reject the null hypothesis.

T-test (two sample) Values

Table 7. *The table below shows the p-values that were obtained from the two sample t-tests that compared week 0 to week 1, week 0 to week 2, and week 1 to week 2 compared to a significance value of 0.05.*

	Ultimate Strength p-value	Young's Modulus p-value
Week 0 compared to Week 1	**0.163**>0.05	**0.440**>0.05
Week 0 compared to Week 2	**0.160**>0.05	**0.641**>0.05
Week 1 compared to Week 2	**0.270**>0.05	**0.247**>0.05

Temperature Summary

The ideal temperature range for human body conditions was between 36.1 and 37.2 °C as stated in the PDS. The temperatures obtained from the bioreactor varied slightly from this ideal range in registering from 31.07 °C to 44.09 °C. The average temperature was 37.55 °C with a standard deviation of 0.56 °C. Throughout the duration of the test, the temperature remained relatively constant within this range and did not experience major fluctuations.

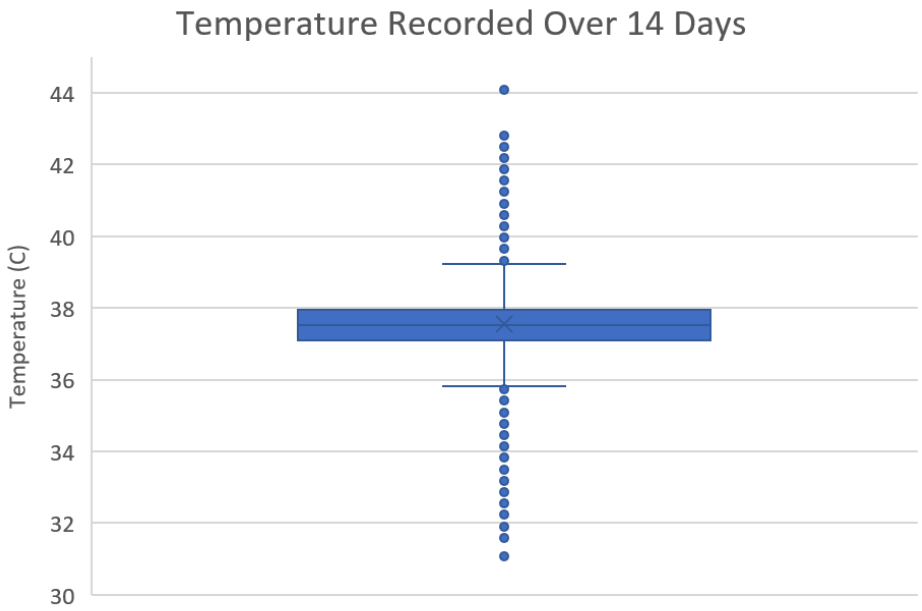


Figure 7. *Above is a box and whisker plot of the temperatures taken from the bioreactor. It shows the frequency of temperatures above and below the mean of 37.5 °C.*

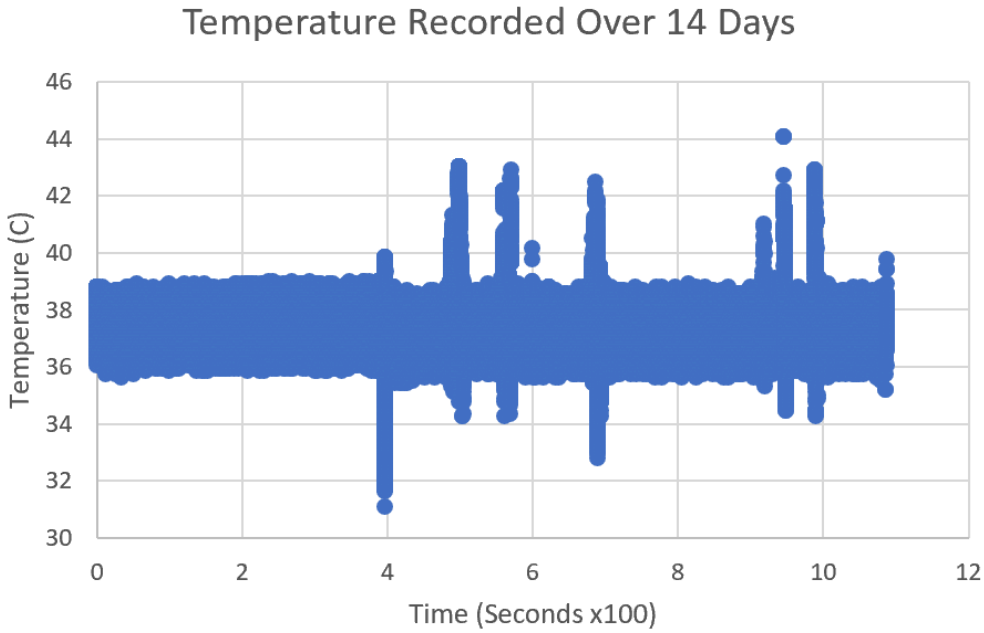


Figure 8. *Above is a scatter plot of all temperature data points vs. time. This plot contains all temperature values obtained over the two-week test period and shows relatively minor fluctuations.*

Comparison of Young's Modulus

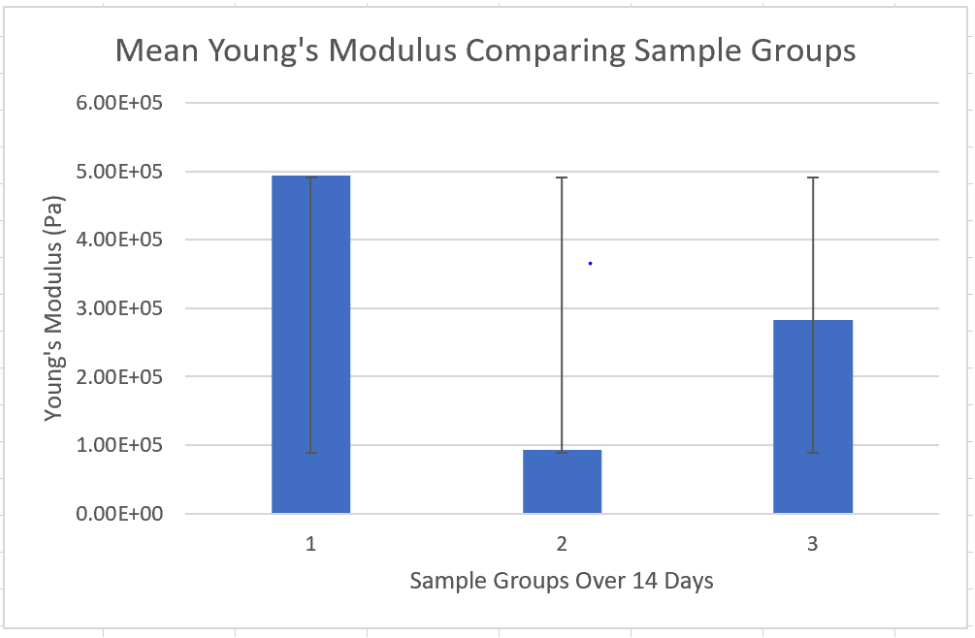


Figure 9. *This bar graph shows how the average Young's Modulus values changed throughout the mechanical strength testing. Error bars calculated from the standard deviation are also shown.*

Discussion

The construction of this bioreactor system has several positive implications. It was able to correctly monitor and adjust the temperature of the solution for the two week testing period with only minor fluctuations. The ideal temperature range determined for human body-like conditions was 36.1 to 37.2 °C. The mean temperature recorded over the two-week test period was 37.5 °C. This is only slightly above the ideal temperature range. The null hypothesis for the MTS testing was that the average Young's modulus and ultimate strengths would remain the same throughout the two-week testing period. The alternate hypothesis stated that the average Young's modulus and ultimate strengths would not remain the same throughout the two-week testing period. The bone graft material was ultimately successful in that it was able to maintain both its Young's modulus and ultimate strength after being subjected to *in vivo* conditions for two weeks. This is evidenced by the fact the p-values for the Young's modulus and the ultimate strength for week zero compared to week one, week zero compared to week two, and week one compared to week two were all larger than the significance value of 0.05. This means that the differences in both the Young's modulus and ultimate strengths between these time periods were not statistically significant. Therefore, the team failed to reject the null hypothesis. Based on these results, it can

be concluded that calcium sulfate functions well as a bone graft and could serve as a good substitute for periodontal bone.

Overall, the entire device system, which includes the bioreactor setup and bone graft material, could be used in further research in order to identify other good bone substitutes that are capable of surviving human body-like conditions for an extended period of time. In order to conduct this research, FDA guidelines regarding medical devices would need to be considered. Also, if any patient trials were involved with this device, researchers would need to first have their plan evaluated by an Institutional Review Board to ensure that risks were minimized for the subjects, that benefits outweigh the risks, and whether or not the groups were given enough information to make an informed decision about participating in the study [38]. This development could ultimately aid surgeons in the repair of bone defects or cavities, decrease the cost of the procedure for the patient, decrease infection rates, and increase the speed at which patients are able to recover.

Conclusions

Summary

The goal of this design project was to develop a synthetic biomaterial that would retain the mechanical properties of bone *in vivo* and fabricate a bioreactor system that was capable of maintaining human body conditions. These goals were accomplished through designing an electronic circuit with a thermistor capable of converting voltage values to temperature values, designing a sample holder that was functional and simple to fabricate, and choosing a biomaterial that reflected many of the same mechanical and biological properties as native periodontal bone. Overall, this device was successful in meeting the criteria detailed in the problem statement. Although the team was not able to physically fabricate the bioreactor or sample holder, the testing data received from past tests show that the bioreactor was capable of maintaining a temperature close to the ideal human range of 36.1 °C to 37.2 °C. Furthermore, the MTS testing data revealed that calcium sulfate's Young's modulus and ultimate strength did not deteriorate over time due to the results of t-test showing that there was not statistically significant difference between the time periods. If anything could have been done differently, the team may have revised the resistor values and equations regarding the electronic circuit to make the thermistor effective within a wider temperature range. Although the bioreactor was never physically tested, preliminary tests showed that the thermistor setup that the team constructed was most accurate within the temperature range it was designed for. However, if the bioreactor solution temperature had exceeded the range the thermistor was designed for, it may not have been as accurate. If the effective range were increased, the thermistor would be able to have a more accurate reading for a larger range of temperatures.

Future Work

Looking forward, there are three main areas where the design outlined in this report could be improved: the bone graft material, electronic circuit design, and sample holder design. Also, in order for this device to be FDA approved, further ISO testing would need to be done [23].

Synthetic Bone Graft Material Selection

Based on the statistical analysis given in the Results section of this report, the calcium sulfate was able to maintain its ultimate strength values and its young's modulus from week zero to week two. This means that it is capable of surviving *in vivo* for two weeks in the provided solution. However, further research will need to be done to determine if it is capable of surviving implantation in the actual human body or body conditions for a longer period of time.

Electronic Circuit

The current thermistor circuit gave the team fairly accurate temperature data from the bioreactor solution. Redesigning the thermistor circuit and code could improve the temperature range output and increase the sensitivity of the reading. This could ultimately give Team Humerus more accurate temperature data. Ideally, the team could design a circuit that functions primarily within the body temperature range and could output voltage differences with very small temperature changes. This may involve the purchase of a more sensitive thermistor.

Sample Holder

While the sample holder was never fabricated, there was a chance that the acrylic material could have experienced mechanical failure due to the laser cutting fabrication or the submersion in the bioreactor solution. Experimenting with different materials and fabrication methods could be extremely useful in determining if acrylic was the correct choice of material to use and if there was a better material substitute.

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coated with collagen or poly (D, L-lactic acid) for alveolar bone augmentation," 2017. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5727736/. [Accessed: 07-Apr-2020].

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Appendices

Appendix A - Product Design Specifications

Periodontal Bone Graft and Bioreactor Design

Function:
Skeletal defects resulting from trauma or other diseases remain a major clinical problem world-wide. Specifically, one of the most common applications for bone grafts are dental implants [39]. These grafts are often used to fill vacant areas in the jaw bone caused by missing teeth. Bone engineering aims to generate viable tissue substitutes in order to provide more readily available bone graft material. Bioreactors help to create the conditions necessary to preserve these synthetic tissues before they can be used. The bioreactor created in this project must be capable of maintaining biological human body conditions in regard to temperature, pH, and salinity for a total of two weeks and must contain a sample holder of some sort for the synthetic bone samples. The bone samples must be made from a material comparable to human bone tissue and must be 20mm deep x 16mm in diameter and must maintain stability similar to native bone for the total duration of two weeks.

Client requirements:

- Bone Graft has to be cylindrical in shape with a 16 mm diameter and 20 mm depth
- Bioreactor has to maintain mechanical stability for at least two weeks
- Bone Graft must be synthetic replacement for the bone
- Bone Graft has to be tested in a physiologically relevant environment

Design Requirements

1. **<u>Physical and Operational Characteristics</u>**

    a. **Performance requirements:**
        i. The bioreactor must be able to maintain a small range of temperature (36.1 to 37.2 degrees Celsius) over the course of two weeks. The bone graft must also be able to withstand the normal wear, tear and use of native bone. This means that it must hold normal loads of human weight and torsion. This includes all daily life activities of a healthy adult individual over his/her lifetime. [42]

    b. **Safety**:
        i. The bone graft has to be made out of a material that is compatible with the human body to avoid being rejected by the host [42]. The Bioreactor must also maintain a temperature that is compatible with the graft but must also be insulated enough so that the user of the bioreactor is not burned or shocked by the exposed heating and electrical elements [43]. In addition, the graft is in a slightly basic solution, so the contents of the bioreactor have to be stored in a manner that prevents prolonged contact with the user [43].

    c. **Accuracy and Reliability:**

        i. In order to simulate accurate values of the human body, the bioreactor has to maintain a narrow range of values. The bioreactor internal temperature must be within 36.1 to 37.2 degrees Celsius [42]. The device must be reliable for a total of two weeks or 336 hours without sacrificing temperature deviation. This accuracy must be maintained to avoid compromising the integrity of the bone graft [44].

    d. **Life in Service:**
        i. The bioreactor has to be able to last at least two weeks (336 hours) without any outcome altering mechanical defects. The bone graft material has to last in vivo for two weeks without any compositional change and also last in the body for two weeks.

    e. **Shelf Life:**

i. The bone graft will be stored at room temperature while not in use or in the bioreactor. The device will be stored in the classroom at room temperature.

f. **Operating Environment:** The graft will be placed inside the human body.
   i. The graft must withstand body temperature for two weeks. (range of 36.1 C to 37.2 C) [45]
   ii. Must be between a pH of 7.35-7.45. [46]
   iii. Salinity- of 9g salt/991g water. [47]
   iv. One cubic inch of the bone graft must withstand 19,000 pounds of force [48]

g. **Ergonomics:** This device will mainly be used in an operating room to be implanted into human bone. The bone graft will need to be sterilized before being implanted and must withstand normal human activity for at least two weeks. It has to travel wherever humans will travel due to the fact that it will be implanted in them.

h. **Size**: The bone graft itself has to be 16mm in diameter and 20 mm deep. The restriction for the bioreactor size is that it has to be able to be placed on the desk in the blue room.

i. **Weight:** There are no strict restrictions on the weight of the device. However, it must be able to be placed and tested on top of a desk.

j. **Materials:** Calcium Sulfate ($CaSO4 * 0.5H2O$) has a degradation rate of 100% when used in biological bone grafts over a 3-6 month period. It is a ceramic that forms an osteoconductive matrix within a bone cavity that allows blood vessels and bone tissue to grow more efficiently. Calcium Phosphates are also a bioceramic that have a similar matrix to bone which enables the graft to be strong. Bioactive glasses consist of mainly silicon dioxide, sodium dioxide, calcium oxide, and phosphorus. Optimum bone grafting bonding occurs between 46-52% silicate composition. Avoid most plastics as there is not enough data and evidence to conclude they are safe for biological use. [49]

k. **Aesthetics, Appearance, and Finish:**

   i. The device has to be made with a Corning jar. The device should be clean and self-contained. This means the device cannot have any exposed wires or components outside the device's general body.

2. **<u>Production Characteristics</u>**

a. **Quantity:** One Bioreactor and 9 synthetic bone samples

b. **Target Product Cost:** $50

3. **Miscellaneous**

a. Standards and Specifications: The bioreactor would be classified as a Class I medical device according to the FDA regulations because it poses little to no harm to the patients [50]. In order for this device to be released to the market, FDA must approve the device in general controls [51]. On the other hand, according to FDA regulations, this bone graft will be classified as a Class II medical device [52]. Since bone grafts are injected, it can pose a moderate risk of illness and injury to the patients such as inflammation, infection, and rejection of the bone graft [53][54]. The bone graft would not be classified as Class III because it does not include therapeutic biologic drugs [55]. The device must be approved by the FDA as a Class II device in both general and special controls before releasing it to the market [56].

b. Customer: Since the targeted customer population are patients who have suffered from bone or joint injuries, the product should deliver effective and efficient healing to their injuries. The bone graft must be able to fill the patient's injury and should have little to no complications such as rejection or contamination. The graft should also be able to heal the patient's injuries quickly.

c. Patient-related concerns: The bioreactor must maintain the sterility of the bone graft as it will be injected into the patient. The prototype bioreactor must match the bodily conditions of the subject and prevent contamination of the graft.

d. Competition:

Pro-Dense [57]

- An injectable regenerative graft comprised of calcium phosphate and calcium sulfate
- Comprised of 75% calcium sulfate and 25% of calcium phosphate
- Is able to be reabsorbed back into the body
- Overall clinical success rate of 86.5% in the Femoral head as well as 78.4% of the patients in the study showed no hip collapse

Bonalive [58]

- Bone granules

- Used to fill bone cavities
- naturally inhibits bacterial growth and stimulates bone formation
- Comprised of 53% SiO2, 23% Na2O, 20% CaO, 4% P2O5

Zimmer: Refobacin® Bone Cement [59]

- high viscosity, antibiotic-loaded bone cement
- unique blend of antibiotics helps to prevent infection
- can be mixed by hand

Stryker [60]

- Vitoss: Synthetic Bone Graft
- features an interconnected, ultra-porous structure that resembles human cancellous bone
- comes in one of three formulations:
    - a foam pack
    - foam strip
    - morsels and blocks
- Highly porous calcium phosphate

Appendix B - Circuit Diagram

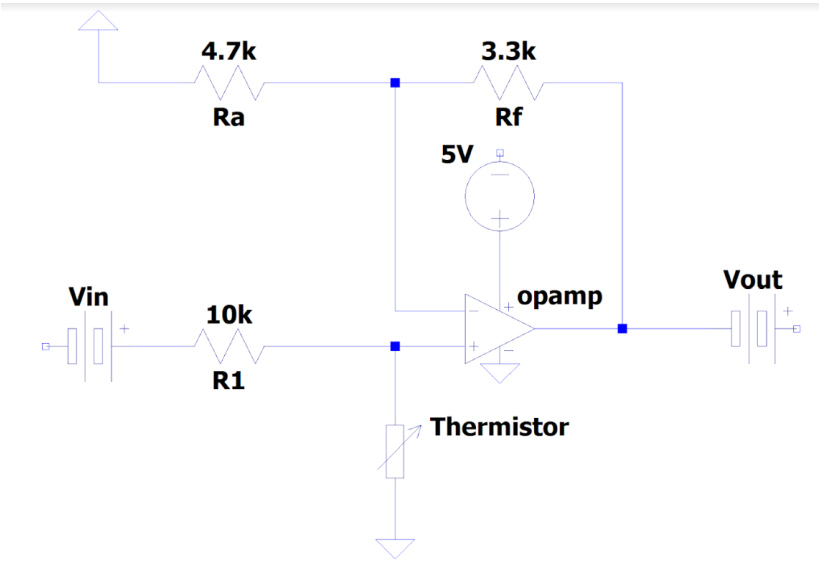


Figure 1. *LTSpice Schematic of Electronic Circuit Design*

$$V_{out} = (1 + \frac{R_f}{Ra})(\frac{R_t}{R_1 + R_t})V_{in}$$

Equation X: Relation between Input and Output Voltage

$$Gain = (1 + \frac{R_f}{Ri}) = 1.70213$$

Equation X: Value of Gain

Appendix C - Block Diagrams

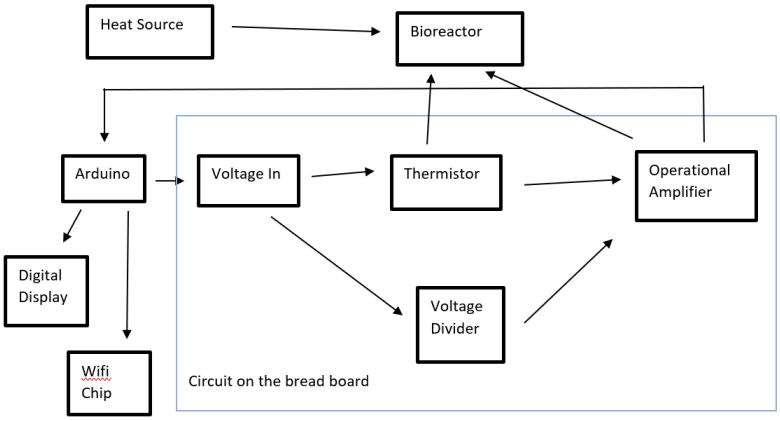


Figure 1. *The diagram above shows the setup of the bioreactor. The Arduino Uno [56] will power the circuit with the thermistor in the bioreactor. The circuit includes a voltage divider and a TLV 271 operational amplifier. The Arduino Uno will read the voltage output from the operational amplifier, convert it into a temperature for the digital display, and will send it to an ESP8266 wifi chip.*

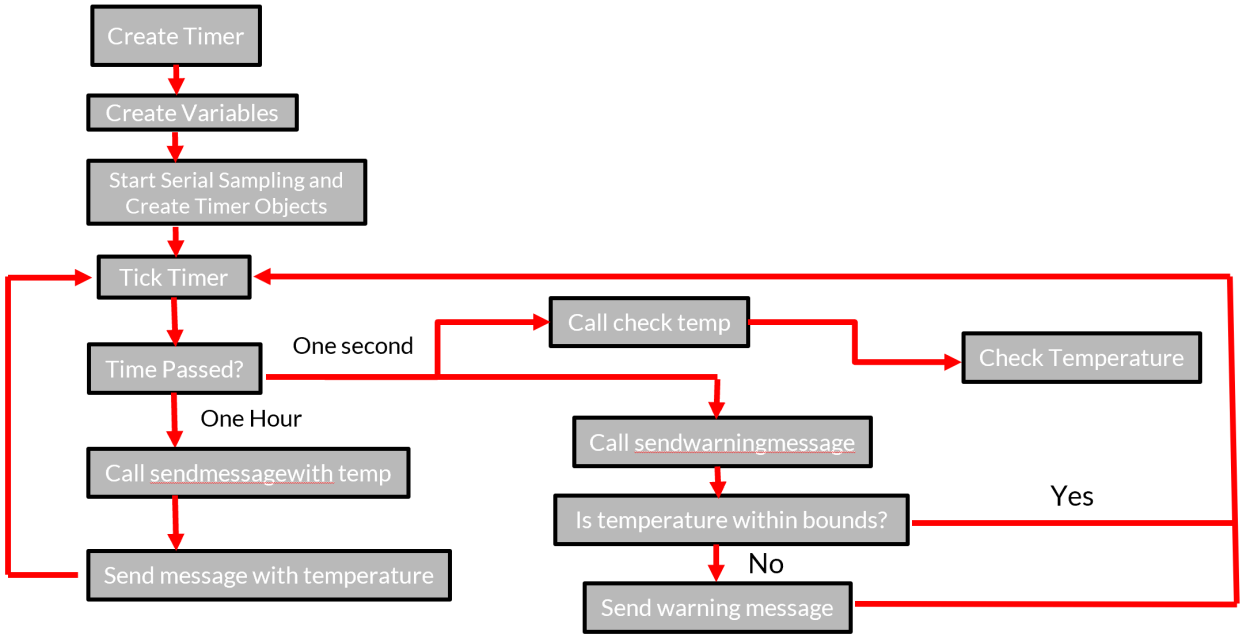


Figure 2. *A software block diagram of the code on the Arduino that is on our thermistor circuit*

Appendix D - Plunger Dimensions

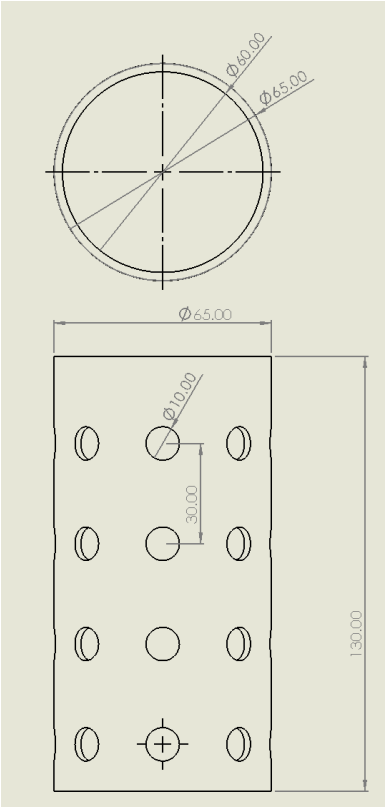


Figure 1. *The top/bottom and side view of the shell casing*

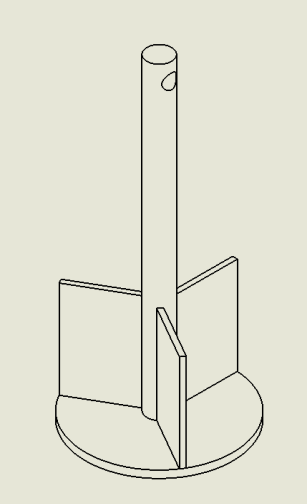


Figure 2. *An isometric view of the first part of the sample holder; this fits inside of the shell and allows for additional modules to be added.*

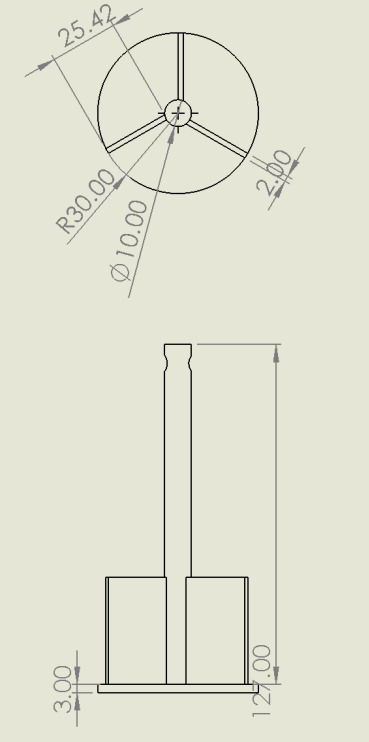


Figure 3. *The top and bottom views of the initial sample holder. This design now includes a hole that enables the user to use a finger to pull the samples out.*

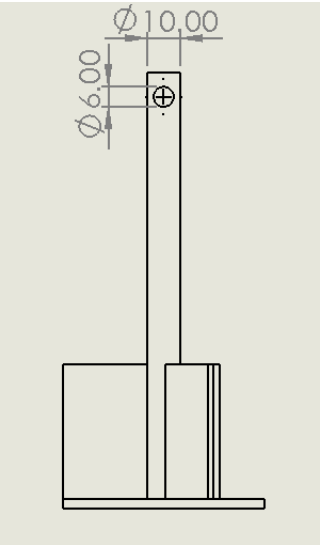


Figure 4. *The side view of sample holder design. The dividers are connected to a center rod which is connected to the base allowing the entire system to be removed as one.*

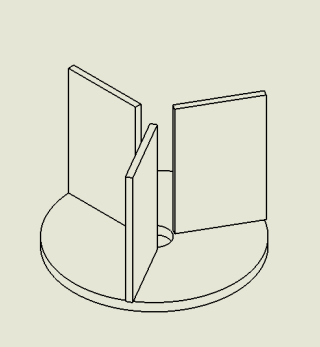


Figure 5. *Isometric view of the addition holder. This part is slid over the rod in the center.*

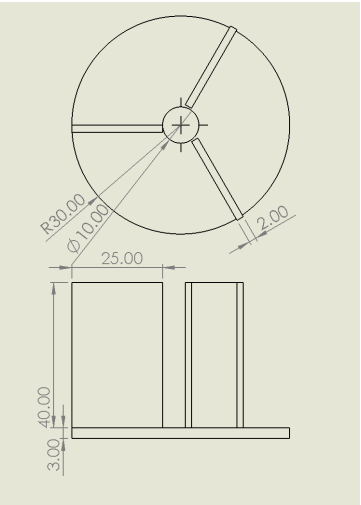


Figure 6. *top and front views of the addition holder. This allows for more sample levels to be added.*

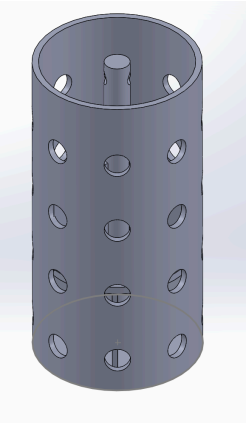


Figure 7. *Isometric view of the entire assembly put together.*

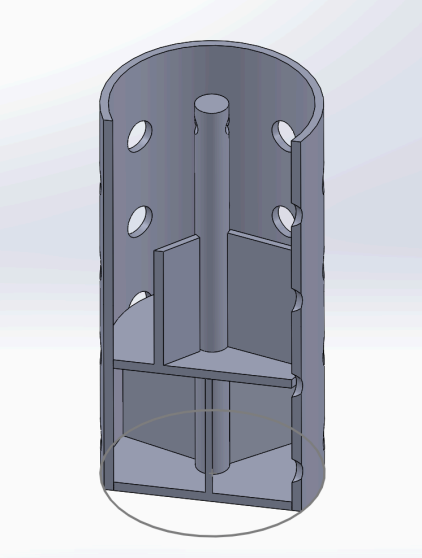


Figure 8. *Cut-away view of the entire assembly. This shows how the samples are contained by the sample holder shell and the baseplate located above them. There is room for a total of nine samples, using two additional sample holders.*

Appendix E - Dimensions for the Dreidel Design

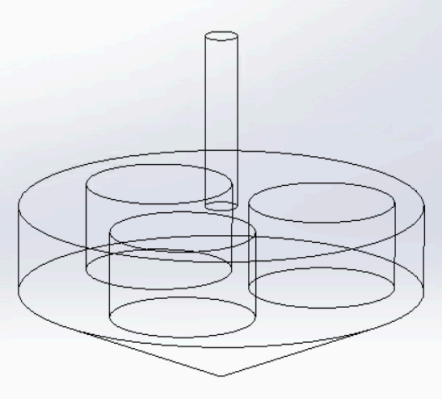


Figure 1. *Wireframe view of the Dreidel Design*

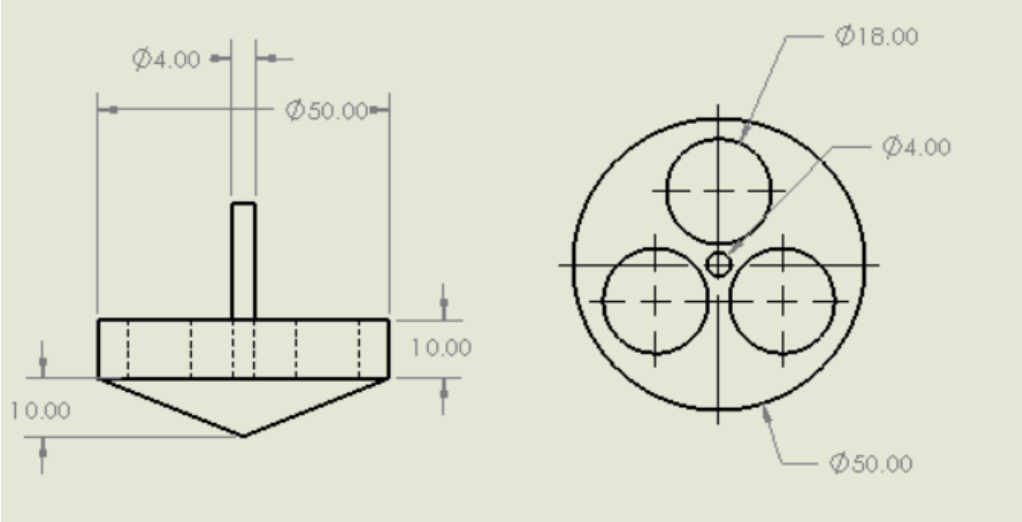


Figure 2. *Measurements of Dreidel Design*

Appendix F - Sample Holder Design Matrix Criteria

Ease of use: Can the design be used to effectively remove and add samples to the bioreactor?

In order to access the bioreactor's contents, one has to stick their hand into the jar through a narrow opening at the top and reach to remove the bone graft sample holder. This means that the hand has to be forced through the opening and come into contact with the fluid/contaminants to reach the desired object. The final design for the bone graft sample holder must have some sort of handle or ergonomic grip for the user to be able to reach and pull out the bone graft samples without disturbing the jar and its contents. This category was given a 20/1000 rating, which is tied with the Ease of Use category and just below the rating of Effectiveness.

Safety: Does the design pose any risk to the user?

The solution within the bioreactor will be hot and may pose a risk to the user when inserting and retrieving the sample holder. The sample holder must be designed to prevent the hot solution from coming into contact with the user. This category was given the weight of only 5/100 because even if the solution does come into contact with the user, it will not be hot enough to pose a large risk.

Durability: Can the design withstand repeated placement into and out of the bioreactor?

In order to retrieve the bone grafts from the bioreactor during and after testing, the entire sample holder will need to be removed through a narrow opening at the top of the corning jar. This

category was given the rating of 10/100 because the sample holder must be durable enough to withstand normal bumps against the lid or repeated handling by the user, but this category is not as important at Ease of Manufacturing or Ease of Use.

Cost: Is the design cost effective and within the budgetary constraints?

The budget for this project is $50. This budget applies to both the materials for the bone graft sample holder and the materials needed to make the synthetic bone graft, so the cost of the sample holder must be kept at a relative minimum. In case of fabrication issues, some of the budget should be left over in case of emergencies. This category was given a weight of 10/100 because it is relatively important to the final design.

Ease of Manufacturing: Can the design be easily fabricated?
The fabrication methods this semester are fairly limited due to the damage in the TEAM Lab. As a result, the only available tools to create the final sample holder are the laser cutter or simple drills. As a result, the manufacturability of the final design is extremely important. This is why this category was given a weight of 30/100, the largest out of all of the criteria.

Effectiveness: Can the design maintain proper placement and keep the bone graft samples submerged?

The device must have some way of either latching onto the bottom of the bioreactor, or be sufficiently heavy to ensure it can stay completely submerged while testing. It also must remain upright and have no risk of tipping over during testing. This category was given the rating of 25/100 because the effectiveness of the bone graft holder is very important in that it is linked to the viability of the testing data.

Appendix G - Biomaterial Chemical Reactions

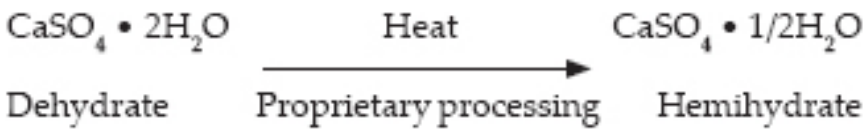


Figure 1. *Chemical Reaction for Calcium Sulfate synthesis [57]*

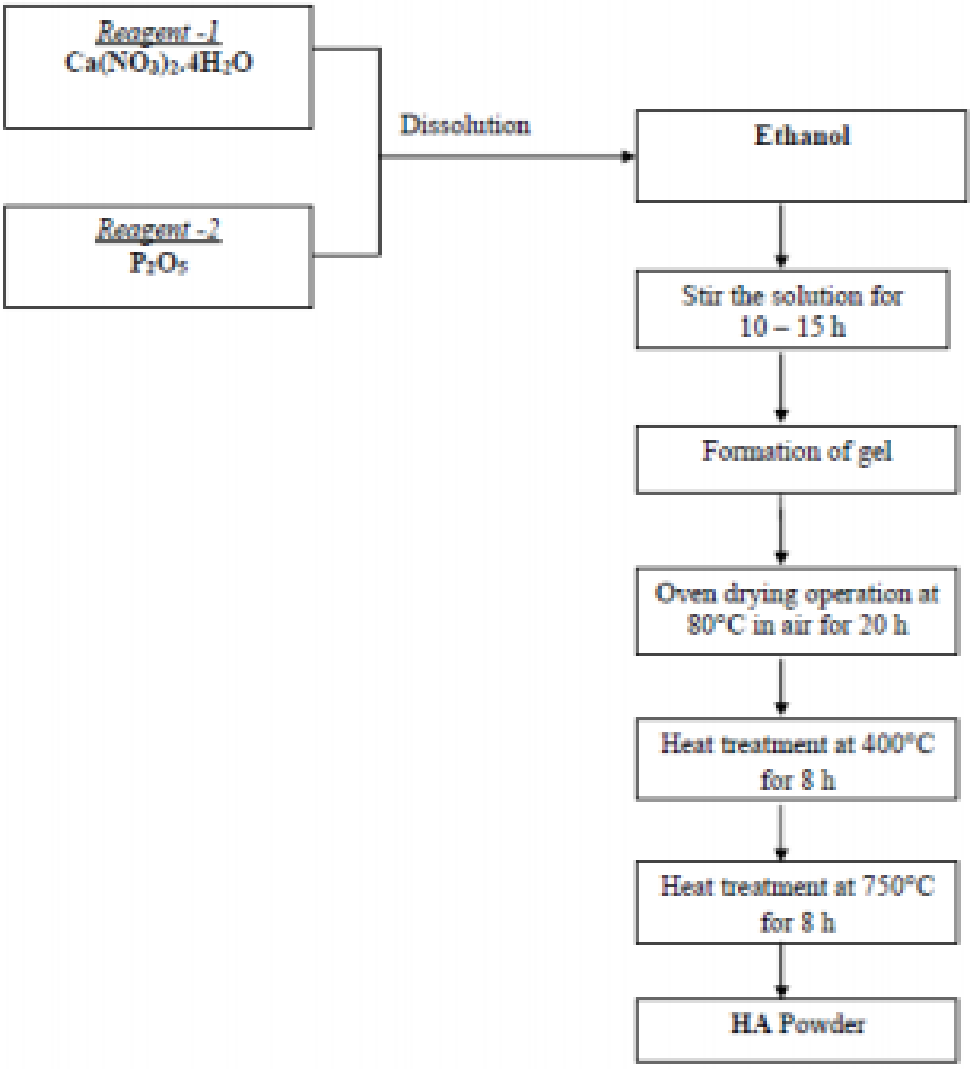


Figure 2. *Flowchart for the synthesis of hydroxyapatite [58]*

$$2NH_4H_2PO_4 + 3CaCO_3 \rightarrow$$

$$Ca_3(PO_4)_2 + NH_3 + 3CO_2 + 3H_2O(1)$$

Figure 3. *Chemical Reaction for synthesis of tricalcium phosphate (TCP) [36]*

Powder	Liquid
I) Polymer: Polymethyl methacrylate/co-polymer (PMMA)	I) Monomer: Methyl methacrylate (MMA)
II) Initiator: Benzoyl peroxide (BPO)	II) Accelerator: N, N-Dimethyl para-toluidine (DMPT)/diMethyl para-toluidine (DMpt)
III) Radio-opacifier: Barium sulphate (BaSO4)/Zirconia (ZrO2)	III) Stabilizer: Hydroquinone
IV) Antibiotics (e.g. Gentamycin)	

Figure 4. *Components of Powder and Liquid MMA for PMMA synthesis [59]*

Appendix H - Biomaterial Design Matrix Criteria

Ease of Fabrication: Can the bone graft be manufactured with the machines and tools available?

Given the time and resources the bone graft needs to be able to be manufactured within a couple days and not require any specialized equipment meaning only using equipment that is available in a standard biochemistry lab. This was weighted the most important category 25/100 because the bone grafts need to actually be manufactured in order to proceed with testing and implantation of the bone graft.

Safety: Does the bone graft material pose any risk to the patient?

The bone graft will be implanted into human paradotal bones. This means that the bone graft material cannot cause harm to the person it is implanted in. This means causing little inflammation or reaction to foreign objects in the body. This was weighted 10/100 because the chosen materials for the matrix have been used as bone grafts previously and are composed of elements that already exist in the human body.

Durability: Can the bone graft withstand normal forces exerted on human bone?

The bone graft must be able to withstand the normal forces placed on it once implanted in the human jaw. This category was given a rank of 20/100. This is tied with the Likeness to Native Bone category because it is pretty important to the overall design.

Cost: Is the design cost effective and within the budgetary constraints of $50?

The budget for this project is $50. This budget is relatively tight, so the cost of the bone graft material must be kept at a relative minimum. This category was given a weight of 15/100 because it is relatively important to the final decision.

Likeness to Native Bone: Does the bone graft material possess similar chemical properties to native bone?

The bone graft material must have similar chemical properties to normal human periodontal bone. This will enable the team to fabricate a bone graft that behaves similar to native bone once implanted in the body. This category was weighted heavily with a rank of 20/100. This is only slightly below the Ease of Manufacturing category because it is very important for the bone graft to be similar to human bone.

Biocompatibility: Is the bone graft compatible with living tissue?

It is important that the bone graft material is biocompatible because it will be directly in contact with native bone and native tissues. This means that it cannot cause harm or damage to surrounding tissues during the entirety of its life inside the body. This was weighted as 10/100 because it is not completely necessary for the material to be biologically inert.

Appendix I - Materials Log

Item	Description	Manufacturer	Part Number	QTY	Cost Each	Total	Link	Justification	Instructor Comm
Biomaterials									
Calcium Sulfate Dihydrate	Powder form	VWR Internation	470300-640 (E	1 (of 500g)	$26.00	$26.00	https://us.vwr.com/store/catalog/static_catalog.jsp?catalog_number=470300-640	Cheapest form of the substance we could find. Used in a variety of synthetic bone grafts.	
ADD YOUR MATERIALS ORDERED HERE (ONLY LIST THE COST FOR THE AMOUNT USED)...						$26.00			
Sample Holder									
Acrylic	Black	Makerspace	N/A	1	$10.75	$10.75	https://making.engr.wisc.edu/mini-mart/#laser-cutter	Purchaseable in the makerspace, able to be used with the laser cutter, and has a high tempature tolerance	
Rod	Stainless Steel	MSC Direct	42440933	1	$2.05	$2.05	https://www.mscdirect.com/product/details/42440933	Used for mounting point in design	
Hex Nut	Stainless Steel, 25 pack	MSC Direct	70976683	1	$5.75	$5.75	https://www.mscdirect.com/product/details/70976683	Used as constraining objects in design	
ADD YOUR MATERIALS ORDERED HERE (ONLY LIST THE COST FOR THE AMOUNT USED)...						$18.55			
					TOTAL:	$44.55			

Figure 1. *The material data sheet that contains all materials used (and planned to use) over the course of the semester and their total costs.*

Table 1. *Electronics Parts List*

Part	Quantity	Cost
Arduino Uno Microcontroller	1	$0
NTCLE413 10K 1 % B3435 K Thermistor	1	$0
10kohm resistor	1	$0
3.3kohm resistor	1	$0
4.7kohm resistor	1	$0
TLV 271 Operational amplifier	1	$0
Connector wires	6	$0
Breadboard	1	$0
ESP8266 Wifi chip	1	$0

Appendix J - Fabrication Protocol for Sample Holder

1. Retrieve acrylic disk of 5mm thick by any size bigger than 60mm x 60mm
2. Use the laser cutter located in the makerspace to cut out three 60mm diameter disks
3. Take one 60mm diameter disk and cut 6 18mm diameter holes in it (through and through)
    a. These holes are spaced out equally from each other radially.
    b. These holes are 5 mm from the outside edge of the 60 mm diameter disk
4. Take another remaining 60mm diameter disk and repeat steps 3a-b but to a hole depth of 1.5mm
5. Take the remaining 60 mm diameter disk and cut a 1.5mm deep 16mm diameter hole at the center of the disk
6. Cut 16mm diameter holes (through and through) in the center of the other two disks
7. Take the threaded nylon rod and place one hex nut on it
8. Flip the threaded rod and place another hex nut on it
    a. Move this hex nut down till it is 30mm from the other hex nut
    b. Place the 60mm diameter place with the 1.5mm depth holes on the rod, hole facing up.
    c. Place a hex nut on the rod and move it to the top of the plate
    d. Secure the plate in place with the bolts

9. Place the other disk with the 6 holes cut all the way through on top of the hex nut that was just placed
10. Place a hex nut on top of the plate to secure it in place
11. Place the remaining disk with the 1.5mm deep center hole on the bottom (away from the holes that are facing, which are up) of the rod

Cut three disks of diameter 60mm from acrylic with laser cutter found in team lab

1. Cut 6 through holes of diameter 18mm in one disk in radial pattern with laser cutter
2. Cut 6 holes approximately 1.5mm deep in other disk in radial pattern with laser cutter
3. Cut one hole in center of third disk of 16mm diameter and 1.5mm deep with laser cutter
4. Place disks on center nylon rod with hex nuts in between
5. Push rod into disk with only one hole to act as the base

Appendix K - Biomaterial Synthesis Protocol

1. Place on appropriate sized gloves
2. Retrieve a 50 mL beaker
3. Retrieve a 100 mL beaker
4. Retrieve a spatula
5. Retrieve 6x (16mm in diameter and 20mm in length) molds for graft
6. Retrieve .5-1000µL eppendorf pipet
7. Retrieve .5-5mL eppendorf pipet
8. Fill 50mL beaker with diH_2O from specific tap
9. Use .5-5 mL eppendorf pipet to transfer 20mL of diH_2O from 50 mL beaker to 100 mL beaker
    a. Set eppendorf pipet to 5mL
    b. Fill to 5 mL from 50mL beaker and then place in 100mL beaker
    c. Repeat 4 times
10. Use the 1,000 µL eppendorf pipet to transfer .147 mL of water from the 50mL beaker to the 100mL beaker
    a. Set eppendorf pipet to 100µL
    b. Transfer 100µL of water from 50mL beaker and transfer to 100mL beaker
    c. Set eppendorf pipet to 47µL
    d. Transfer 47µL of water from 50mL beaker to 100mL beaker
11. Retrieve calcium sulfate di-hydrate material in the powdered form and go to the weigh station
12. Turn on scale
13. Retrieve and place weigh boat onto the scale
14. Zero the scale

15. Place 42g of the calcium sulfate powder in the weigh boat
16. Place the measured calcium sulfate into tho 100mL beaker
17. Stir the mixture in the 100mL beaker for approximately 3 minutes [60]
18. Let the material rest in the 100mL beaker for 2 minutes[60]
19. Place six molds on counter and fill the molds with the mixture from the 100mL beaker
    a. There will be material left over in the 100mL beaker
    b. Dispose of this excess into the trash
20. Place molds onto vibrating table
    a. Place the vibrating table at 6000 rpm for 5 minutes[60]
21. Remove molds from table and let set standing (on end cap) up for 30 minutes[60]
22. Retrieve hot plate
    a. Set hot plate to 90 degrees celsius [60]
    b. Place molds into 250mL beaker standing up [60]
    c. Place watch glass on top of beaker
    d. Let molds heat for 20 minutes or longer if not dry at the end time of 10 minutes
        i. If not try check every 5 minutes
23. Remove beaker from hot plate
24. Remove and replace gloves
25. Turn on the UV light in the biological safety cabinet (BSC) for 15 minutes
26. After 15 minutes, open the sash and start the blower and wait for 15-20 min
27. Spray down surfaces with 70% ethanol
28. Retrieve plastic board
29. Wipe down board with 70% ethanol
30. Wipe down plastic sides of graft with 70% ethanol
31. Remove plastic from grafts and place onto plastic board
32. Place plastic board into BSC
33. Close the sash and turn on UV light for 15 minutes
34. Open sash and remove plastic board
35. Sample synthesis and sterilization is complete
36. Repeat steps 1-35 to create a total of 12 molds
37. Place molds into bioreactor, selected the best 9 molds out of the 12 created

Appendix L - Calculations for Synthesis

- The graft dimensions are 16mm in diameter and 20mm in length
    - This yields a volume of 4021.23mm^3

- The ratio used for the mixture of water to calcium sulfate is .67mL of water to 1g of powder [60]. Using the number above we can use 4.021mL of water as our starting point
- $4.021mL/.67mL/g = 6\ g\ of\ powder$
- This means that we need 4.021mL of water for 6 g of powder
    - This can be assumed roughly to be the volume of one graft
        - There will be excess, but that is assumed that it will not be possible to effectively transfer all the contents of the mixture to the graft mold
- We are creating 6 molds, but will make the number we calculate for 7 molds to make sure that there is excess so that our are able to get the right amount into the mold and still leave some material in the mixing container
- 6g x 7 = 42g
- 4.021mL x 7 = 28.147mL

Appendix M - Thermistor Datasheet

Table 3

PART IDENTIFICATION	R_{25}		$B_{25/85}$	
	kΩ	± %	K	± %
NTCLE413 10K 1 % B3435 K	10	1	3435	1.0

RESISTANCE VALUES AT INTERMEDIATE TEMPERATURES

TEMPERATURE (°C)	R_T (Ω)	R_T/R_{25}	R-TOL. (± %)	α (%/K)	T-TOL. (± °C)	$R_{MIN.}$ (Ω)	$R_{MAX.}$ (Ω)
-40.0	190 953	19.095	4.24	-5.46	0.78	182 848	199 057
-35.0	145 953	14.595	3.93	-5.30	0.74	140 213	151 693
-30.0	112 440	11.244	3.63	-5.14	0.71	108 354	116 526
-25.0	87 285	8.7285	3.35	-4.99	0.67	84 364	90 206
-20.0	68 260	6.8260	3.07	-4.85	0.63	66 164	70 355
-15.0	53 762	5.3762	2.80	-4.71	0.60	52 254	55 270
-10.0	42 636	4.2636	2.55	-4.57	0.56	41 549	43 723
-5.0	34 038	3.4038	2.30	-4.44	0.52	33 254	34 822
0.0	27 348	2.7348	2.07	-4.31	0.48	26 783	27 913
5.0	22 108	2.2108	1.84	-4.19	0.44	21 702	22 515
10.0	17 979	1.7979	1.62	-4.08	0.40	17 689	18 270
15.0	14 706	1.4706	1.40	-3.96	0.35	14 499	14 912
20.0	12 094	1.2094	1.20	-3.86	0.31	11 949	12 239
25.0	10 000	1.0000	1.00	-3.75	0.27	9900.0	10 100
30.0	8310.8	0.83108	1.19	-3.65	0.33	8211.7	8409.8
35.0	6941.1	0.69411	1.38	-3.55	0.39	6845.5	7036.7
40.0	5824.9	0.58249	1.56	-3.46	0.45	5734.1	5915.6
45.0	4910.6	0.49106	1.73	-3.37	0.51	4825.6	4995.7
50.0	4158.3	0.41583	1.90	-3.28	0.58	4079.2	4237.3
55.0	3536.2	0.35362	2.06	-3.20	0.65	3463.2	3609.2
60.0	3019.7	0.30197	2.22	-3.12	0.71	2952.5	3086.8
65.0	2588.8	0.25888	2.38	-3.04	0.78	2527.3	2650.4
70.0	2228.0	0.22280	2.53	-2.96	0.85	2171.7	2284.3
75.0	1924.6	0.19246	2.67	-2.89	0.92	1873.1	1976.0
80.0	1668.4	0.16684	2.81	-2.82	1.00	1621.5	1715.3
85.0	1451.3	0.14513	2.95	-2.75	1.07	1408.5	1494.2
90.0	1266.7	0.12667	3.08	-2.69	1.15	1227.7	1305.8
95.0	1109.2	0.11092	3.21	-2.62	1.22	1073.6	1144.8
100.0	974.26	0.097426	3.34	-2.56	1.30	941.74	1006.8
105.0	858.33	0.085833	3.46	-2.50	1.38	828.62	888.04

Appendix N - Thermistor, Circuit, and Microcontroller Testing Protocols

Test 1: Thermistor

Purpose: This test evaluates how accurately the thermistor can detect the temperature of a water bath.

Materials:

- Electronic circuit with thermistor
- 3 water baths of three different temperatures
    - 23 ^{0}C, 27 ^{0}C, and 52 ^{0}C
- Computer to run Arduino code

Protocol:

1. Take temperature reading of water with thermometer
2. Run Arduino code on computer
3. Place thermistor into water bath
4. Wait for temperature reading on serial output to stabilize
5. Record measured temperature
6. Repeat 3 times for each temperature

Test 2: Electronics

Purpose: This test evaluates if the circuit can output the correct voltages.

Materials:

- Completed circuit with thermistor
- Computer with Arduino code
- Water baths of 25 ^{0}C and 36 ^{0}C

Protocol:

1. Calculate predicted voltage outputs for temperatures of 25 ^{0}C and 36 ^{0}C
2. Place thermistor in water bath
3. Obtain voltage value from serial readout
4. Record and compare to predicted value

Test 3: Microcontroller Code

Purpose: This test evaluates whether the Arduino code is functioning properly to correctly convert voltage readings to temperature.

Materials:

- Computer with Arduino code
- Completed circuit

Protocol:

1. Manually input voltage values of 4.225 and 2.837 V (corresponding to 25 ^{0}C and 45 ^{0}C) into program
2. Obtain outputted temperature values and use them to compare to the expected values

Appendix O - Arduino Code

```
#include <timer.h>
#include <math.h>

// BME 201 lab code

auto timer = timer_create_default(); // creates timer
String GscriptID = "" ;// where google string will be placed

float rawoutput;
float voltageoutput;
float upperbound;
float lowerbound;
float r1;
float rf ;
float ra ;
float vin = 5.0;
float a;
float y;
float x;
float z;
float temp;


bool checktemp(void *){ // updates temp value every 5 seconds
  rawoutput = analogRead(A0);
  voltageoutput = (rawoutput/1024.0)*5.0;
  r1 = 10000;
  rf = 3300;
  ra = 4700;
  vin = 5.0;
  a = (1.0+(rf/ra));
  y = (voltageoutput/(vin*a));
  x = ((y*r1)/(1.0-y));
  z = log((1.0/23139.0)*x);
```

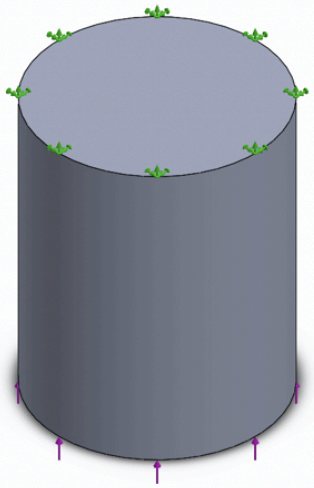
Figure 1. *The first part of the Arduino code to interpret the voltage output from the circuit.*

```
  temp = (z/-0.034);
  Serial.println(rawoutput, 6);
  Serial.println(voltageoutput, 6);
  Serial.println(temp,6);

  return true; // repeats
}


bool sendmessagewithtemp(void *){
  Serial.println(GscriptID + "The current temp is " + String(temp));
  return true; // repeats
}

bool sendwarningmessage(void *){
  if(temp > upperbound){
      Serial.println(GscriptID + "WARNING TEMP IS OVER BOUND" + String(temp));
  }
  if(temp < lowerbound){
      Serial.println(GscriptID + "WARNING TEMP IS OVER BOUND" + String(temp));
  }
  return true; //repeats
}
void setup() {

  Serial.begin(9600);
  timer.every(1000,checktemp); // checks the temp every 5 seconds
  timer.every(3600000, sendmessagewithtemp); // sends temp every hour
  timer.every(1000, sendwarningmessage);

}
```

Figure 1.2 *The second part of the Arduino code to interpret the voltage output from the circuit.*

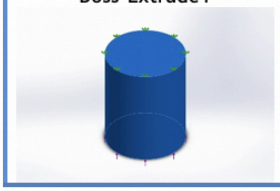
Appendix P - SolidWorks Testing Data

Model Information

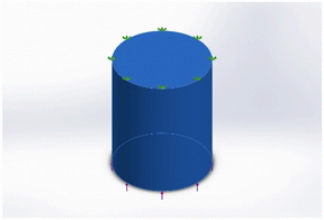


Model name: sampletest (1)
Current Configuration: Default

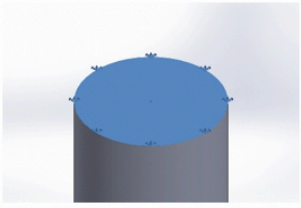
Solid Bodies

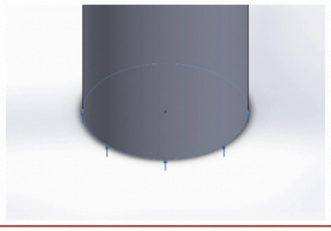
Document Name and Reference	Treated As	Volumetric Properties	Document Path/Date Modified
Boss-Extrude1 	Solid Body	**Mass:0.00932927 kg** **Volume:4.02124e-06 m^3** **Density:2,320 kg/m^3** **Weight:0.0914269 N**	**C:\Users\ethan\Downloads \sampletest (1).SLDPRT** Mar 8 18:14:34 2020

Material Properties

Model Reference	Properties	Components
	**Name:** Calcium Sulfate **Model type:** Linear Elastic Isotropic **Default failure criterion:** Max von Mises Stress **Yield strength:** 1.77e+07 N/m^2 **Tensile strength:** 3e+07 N/m^2	SolidBody 1(Boss-Extrude1)(Part1)

Loads and Fixtures

Fixture name	Fixture Image	Fixture Details
Fixed-1		**Entities:** 1 face(s) **Type:** Fixed Geometry

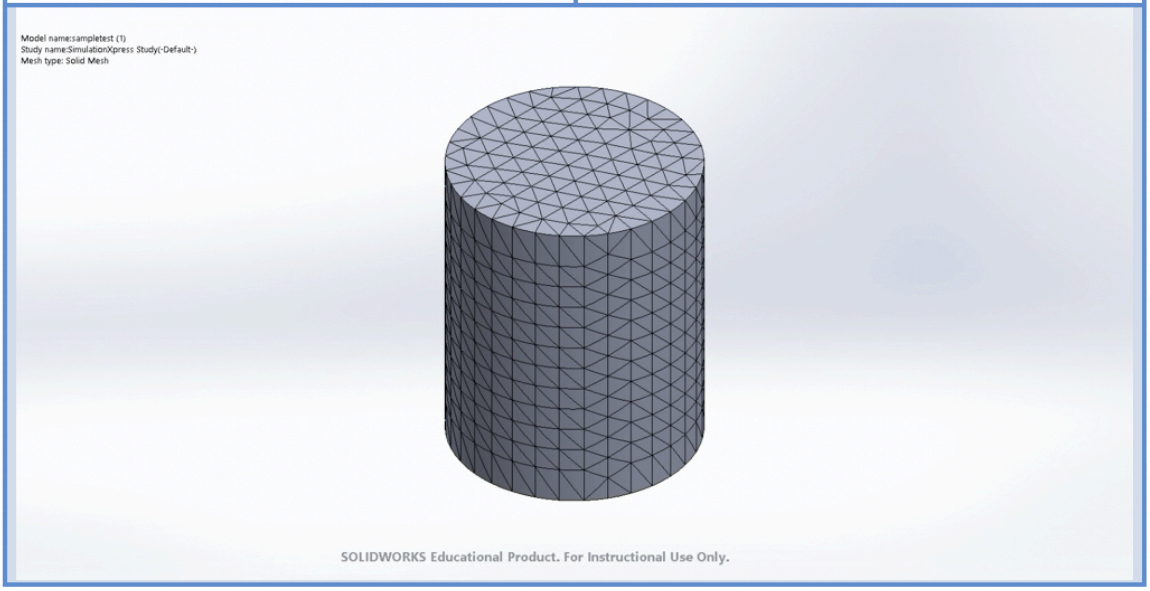
Load name	Load Image	Load Details
Force-1		**Entities:** 1 face(s) **Type:** Apply normal force **Value:** 10,000 N

Mesh information

Mesh type	Solid Mesh
Mesher Used:	Standard mesh
Automatic Transition:	Off
Include Mesh Auto Loops:	Off
Jacobian points	4 Points
Element Size	1.59086 mm
Tolerance	0.0795432 mm
Mesh Quality Plot	High

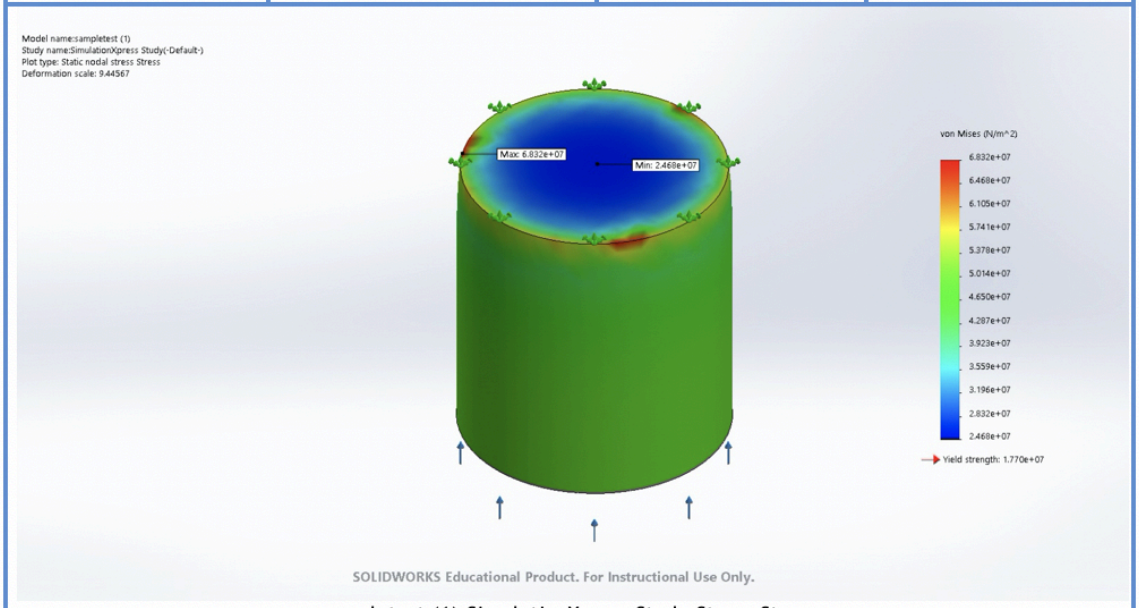
Mesh information - Details

Total Nodes	10467
Total Elements	6970
Maximum Aspect Ratio	3.3306
% of elements with Aspect Ratio < 3	99.9
% of elements with Aspect Ratio > 10	0
% of distorted elements(Jacobian)	0
Time to complete mesh(hh:mm:ss):	00:00:01
Computer name:	WIN-F151RQ2



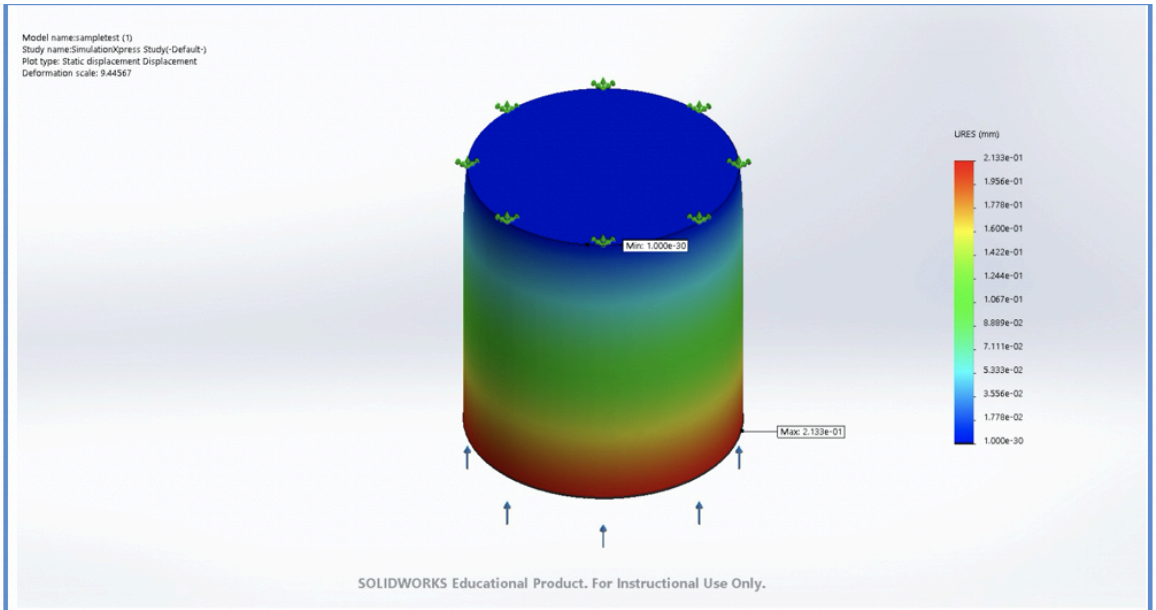
Study Results

Name	Type	Min	Max
Stress	VON: von Mises Stress	2.468e+07 N/m^2 Node: 7550	6.832e+07 N/m^2 Node: 10402



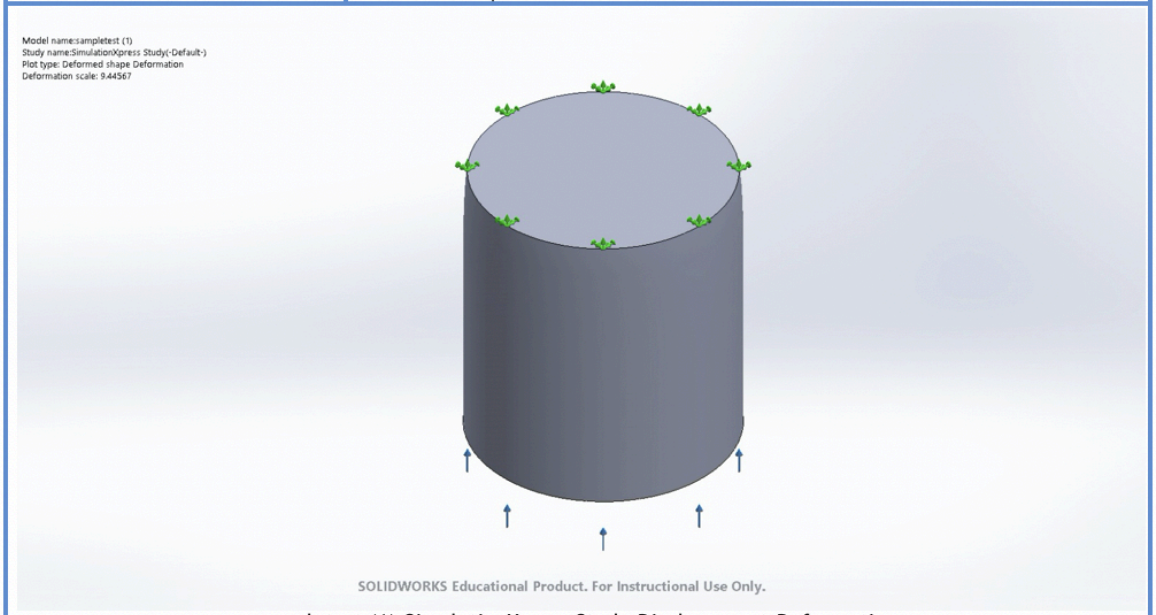
sampletest (1)-SimulationXpress Study-Stress-Stress

Name	Type	Min	Max
Displacement	URES: Resultant Displacement	0.000e+00 mm Node: 1	2.133e-01 mm Node: 175



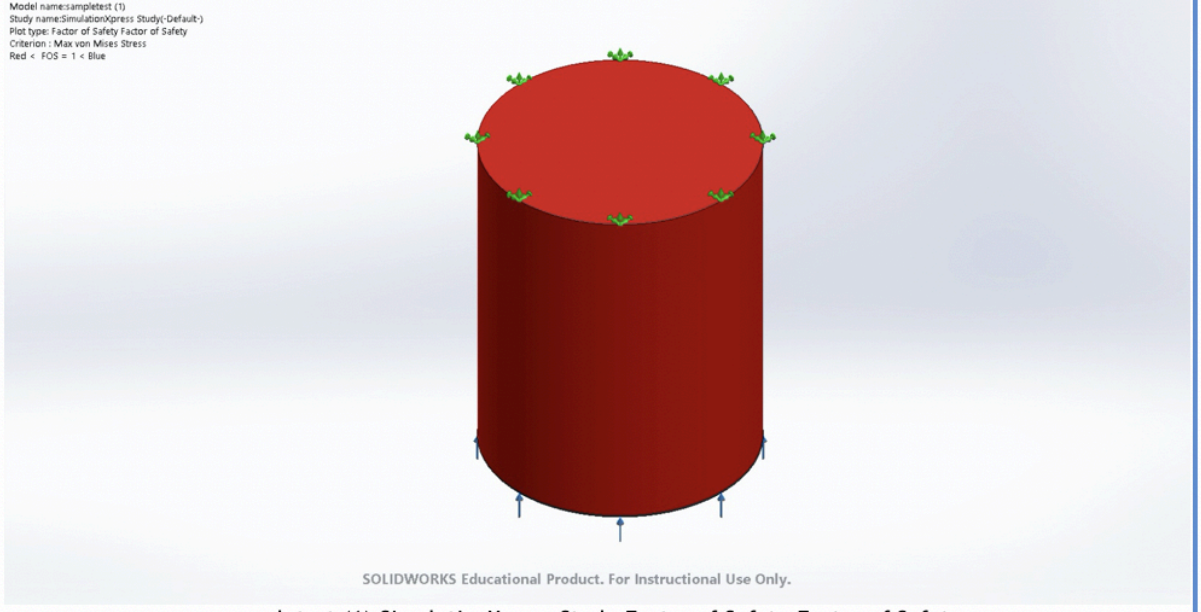
sampletest (1)-SimulationXpress Study-Displacement-Displacement

Name	Type
Deformation	Deformed shape



sampletest (1)-SimulationXpress Study-Displacement-Deformation

Name	Type	Min	Max
Factor of Safety	Max von Mises Stress	2.591e-01 Node: 10402	7.171e-01 Node: 7550



sampletest (1)-SimulationXpress Study-Factor of Safety-Factor of Safety

Figure 1. *Solidworks simulation testing data. It shows that the test sample fails in compression as required by the client.*

Appendix Q - MTS Testing Protocol

Purpose:

Team Humerus will be utilizing an MTS machine to create stress vs strain plots of calcium sulfate bone grafts. Team Humerus can then use these graphs to obtain important information about the mechanical properties of calcium sulfate such as the material's ultimate strength, fracture point, and Young's modulus of each given sample. After acquiring these pieces of data, the values from week 0 can be compared to weeks 1 and 2 using a two-sample t-test for statistical analysis.

Hypotheses:

Null hypothesis (H_o): The average elastic modulus and ultimate strength of the calcium sulfate samples from week 0 will be the same as the average elastic modulus when compared to week 1 and week 2.

Alternative hypothesis (H_a): The average elastic modulus and ultimate strength of the calcium sulfate samples from week 0 will not be the same as the average elastic modulus and ultimate strength when compared to week 1 and week 2.

Preparation of Samples:

1. Obtain samples from bioreactor
2. Remove samples from sample holder
3. Sand all samples to ensure each top is flat
4. Measure each sample's length and diameter with a caliper and record
5. Weigh each sample and record
6. Note any differences in measurements and make physical observations for defects

Preparation of the MTS Machine:

1. Become familiar with the general setup of the machine and identify the red emergency stop button
2. Identify the max load for calcium sulfate:
3. Identify the appropriate load cell based on this value. In this case, the proper load cell will be 10,000 N
4. Attach the fixture to the clevis of the machine
    a. Insert fixture into top hole of clevis
    b. Insert pin into side/front hole of clevis and fixture
    c. Tighten collar with fingers
    d. Finish tightening collar with wrench, being careful not to tighten too much
5. Set Safety travel limit switch to prevent the top and bottom fixtures from colliding together
    a. Use handset to move compression plates close together
    b. Use fine movement wheel to ensure the plates do not touch each other, leave about a 3mm gap
    c. Loosen bottom stop thumb screw and slide it up until it reaches crosshead locator
    d. Use the fine movement on handset to test the stop
6. Enter parameters in software
    a. Open software TestSuite and choose "simplified compression"
    b. Find the Monitor tab and enter sample diameter by clicking in the box

Running the Test and Collecting Data:

1. Insert previously prepared sample
    a. Center sample between plates
    b. Use fine movement wheel to cause the plate to barely touch the sample
2. Open TestSuite

   a. Click file new → test → from template → select the test needed for your experiment and enter the Sample Parameters
   b. Choose “simplified compression”
   c. Find the Monitor tab and enter sample diameter by clicking in the box
3. Slightly load sample
   a. Unlock the Crosshead on the handset and move the Crosshead towards the sample until it just barely loads the sample
   b. Zero the System on the MTS computer screen (right click on Load and then Crosshead → Zero Signal)
4. Click Play software - Run until the curve flattens, failure, or load limit
   a. Be sure to watch data collection for any issues
5. Click the “Play” button to start the test
6. Watch carefully to ensure the load does not exceed limits on the load cell and fixture
7. If needed, use stop icon in software or machine emergency stop to end the test\
8. Save the raw data that is collected

Appendix R - Raw Young’s Modulus and Ultimate Strength Data

Table 1. *The table below shows the Young’s modulus and Ultimate Strength for each bone graft. The run number (ex. Run 1) corresponds to the same bone graft that is tested over time.*

	Run	Young's Modulus	Ultimate strength
Week 0	2	8.2821e5	5.2197e5
	3	1.5976e6	8.6819e5
Week 1	1	4.5007e4	1.0183e5
	2	6.4997e4	1.5403e4
	3	1.6761e5	6.1053e4
Week 2	1	5.1615e4	1.0251e4
	2	3.4593e4	2.8303e4
	3	4.4858e4	2.6712e4

Appendix S - MTS testing Graphs of Stress Strain Curves

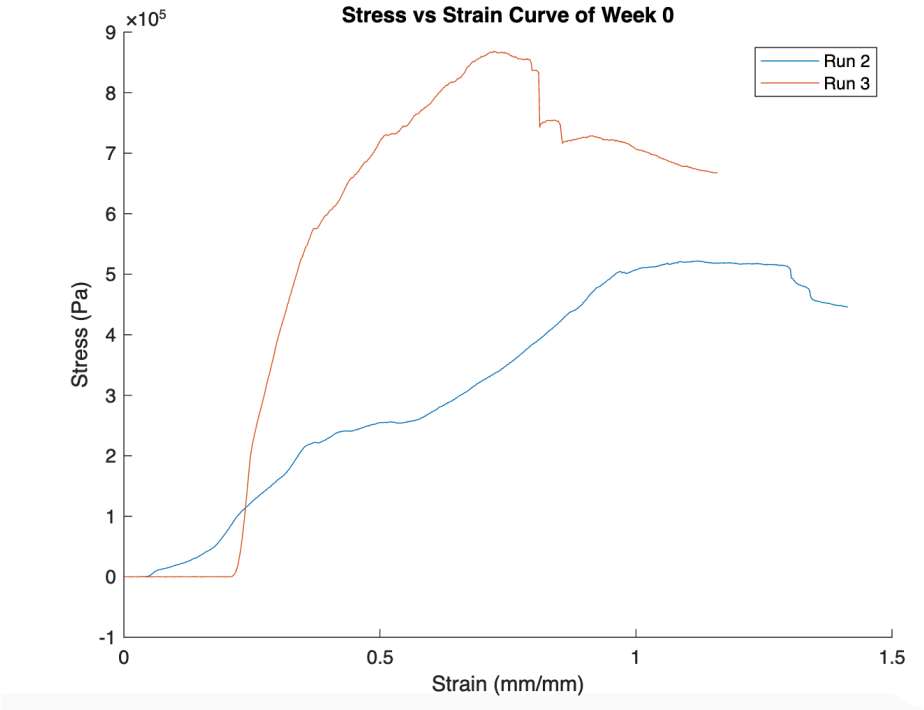


Figure 1: MTS curve for Week 0

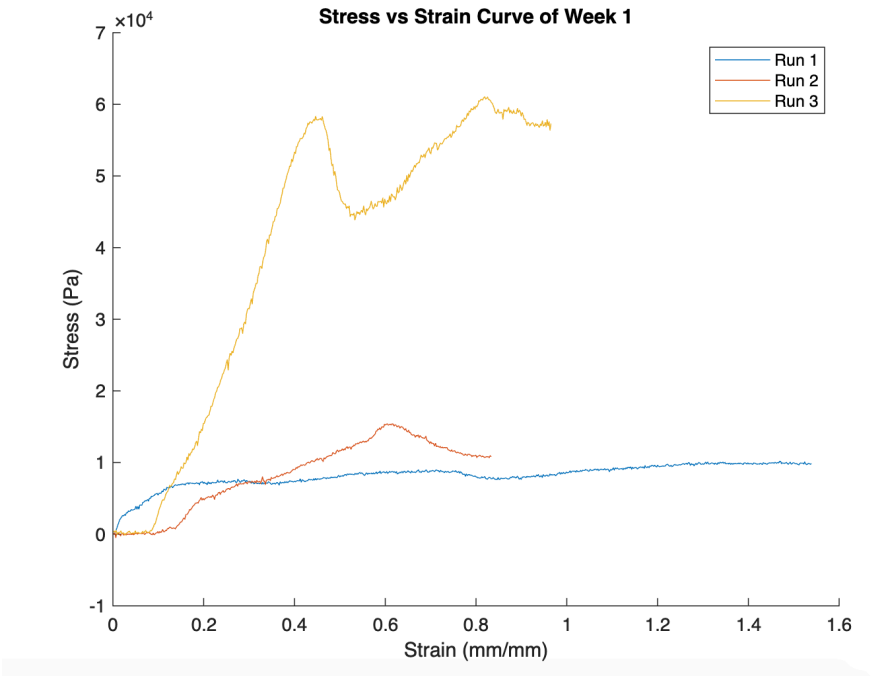


Figure 2: MTS curve for Week 0

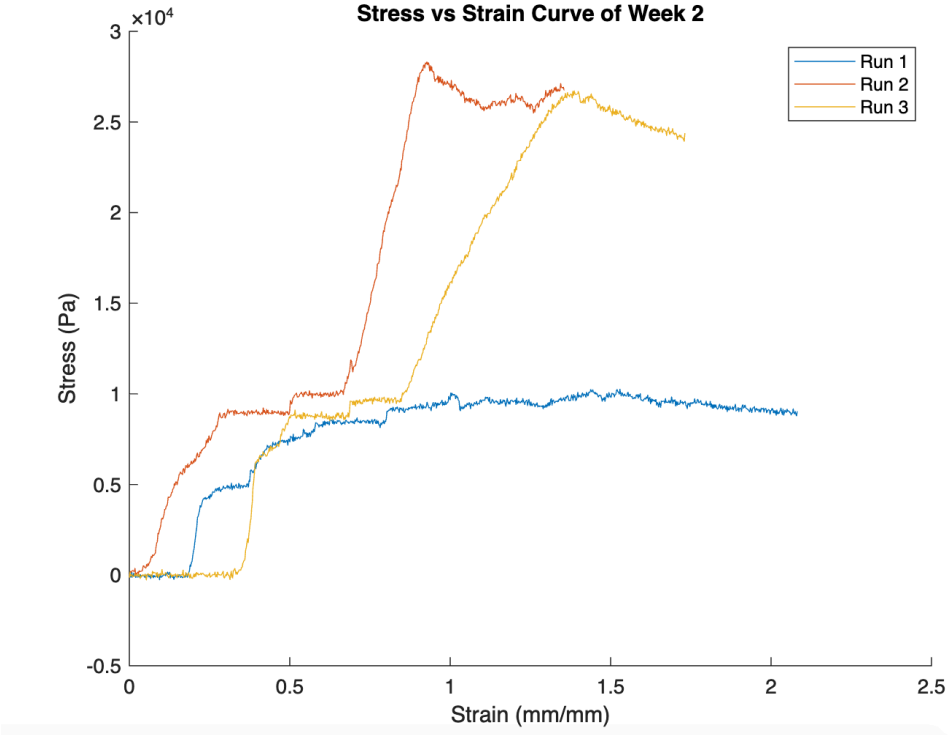


Figure 3: MTS Curve for Week 2

Appendix T - MATLAB script for MTS testing

```
clc

testing0_2data = load('run2t0.txt');
testing0_3data = load('run3t0.txt');
testing1_1data = load('run1t1.txt');
testing1_2data = load('run2t1.txt');
testing1_3data = load('run3t1.txt');
testing2_1data = load('run1t2.txt');
testing2_2data = load('run2t2.txt');
testing2_3data = load('run3t2.txt');
t02_strain = testing0_2data(:,1);
t03_strain = testing0_3data(:,1);
t11_strain = testing1_1data(:,1);
t12_strain = testing1_2data(:,1);
t13_strain = testing1_3data(:,1);
t21_strain = testing2_1data(:,1);
t22_strain = testing2_2data(:,1);
t23_strain = testing2_3data(:,1);

t02_stress = ((testing0_2data(:,2))/.000804);
t03_stress = ((testing0_3data(:,2))/.000804);
t11_stress = ((testing1_1data(:,2))/.000804);
t12_stress = ((testing1_2data(:,2))/.000804);
t13_stress = ((testing1_3data(:,2))/.000804);
t21_stress = ((testing2_1data(:,2))/.000804);
t22_stress = ((testing2_2data(:,2))/.000804);
t23_stress = ((testing2_3data(:,2))/.000804);
```

```
%figure1
figure(1);
hold on
plot(t02_strain,t02_stress);
plot(t03_strain,t03_stress);
xlabel('Strain (mm/mm)')
ylabel('Stress (Pa)')
legend('Run 2','Run 3')
title("Stress vs Strain Curve of Week 0")
hold off

%figure2
figure(2);
hold on
plot(t11_strain,t11_stress);
plot(t12_strain,t12_stress);
plot(t13_strain,t13_stress);
xlabel('Strain (mm/mm)')
ylabel('Stress (Pa)')
title("Stress vs Strain Curve of Week 1")
legend('Run 1','Run 2','Run 3')
hold off


  % linear regressions of lines (youngs mod)

  pt02 = polyfit(t02_strain(104:179),t02_stress(104:179),1) ;% linear
   region stops around x = .368
  pt03 = polyfit(t03_strain(1:175),t03_stress(1:175),1); % linear
   reagion stops around x = .3657
  pt11 = polyfit(t11_strain(1:64),t11_stress(1:64),1); % linear region
   stops around x = .1124
  pt12 = polyfit(t12_strain(64:100),t12_stress(64:100),1);% lienar
   region stops around x = .1964
  pt13 = polyfit(t13_strain(39:215),t13_stress(39:215),1); % linear
   region stops around x = .4338
  pt21 = polyfit(t21_strain(90:130),t21_stress(90:130),1); % linear
   region stops around x = .2588
  pt22 = polyfit(t22_strain(21:134),t22_stress(21:134),1); % linear
   region stops around x = .438
  pt23 = polyfit(t23_strain(169:239),t23_stress(169:239),1); % linear
   region stops around x = .2925

  % average linear regression of lines

  ym0=[pt02,pt03]
  ym1=[pt11,pt12,pt13]
  ym2=[pt21,pt22,pt23]
  %value testing
```

```
figure(3);
hold on
plot(t21_strain,t21_stress);
plot(t22_strain,t22_stress);
plot(t23_strain,t23_stress);
xlabel('Strain (mm/mm)')
ylabel('Stress (Pa)')
title("Stress vs Strain Curve of Week 2")
legend('Run 1','Run 2','Run 3')
hold off

%maximums (ultimate strengths)

max_t02 = max(t02_stress);
max_t03 = max(t03_stress);
max_t11 = max(t11_stress);
max_t12 = max(t12_stress);
max_t13 = max(t13_stress);
max_t21 = max(t21_stress);
max_t22 = max(t22_stress);
max_t23 = max(t23_stress);

%averages of maximums per week

ult0 = [max_t02,max_t03]
utl1 = [max_t11,max_t12,max_t13]
ult2 = [max_t21,max_t22,max_t23]




    %ult w0-w1
[ultw01,ultw01x] = ttest2(ult0,utl1);
ultw01x;
    %ult w0-w2


[ultw02,ultw02x]=ttest2(ult0,ult2);
ultw02x;
    %ym w0-w1
[ymw01,ymw01x]=ttest2(ym0,ym1);
ymw01x;
    %ym w0-w2
[ymw02,ymw02x]=ttest2(ym0,ym2);
ymw02x;
```