THE USE OF LAYERED FREEFORM FABRICATION TECHNOLOGIES TO PRODUCE TISSUE ENGINEERING SCAFFOLDS FOR SKULL PATCHES

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Abstract

Congenital skull defects in infants are difficult to correct using metal plates due to the growth of the skull. Tissue engineering of bone patches could be the answer to help such patients. Custom scaffolds have been designed based on Computed Tomography (CT) images of the patient's skull. An in-house developed single screw extruder, casting and a commercial laser cutter has been evaluated in the fabrication of pure polycaprolactone (PCL) scaffolds as well as PCL mixed with hydroxyapatite (HA) scaffolds. Evaluation criteria for each process included the ability to maintain an optimal pore size for cells to proliferate, inclusion of micro surface properties for cell adhesion, incorporation of hydroxyapatite, and ability to maintain desired shape. The mechanical properties of the fabricated scaffolds will be presented in this paper as well as initial cell seeding results with human adipose-derived adult stem (hADAS) cells.

Background

Tissue engineering involves the combination of cells, organic, and inorganic materials in an effort to repair a biological component or function. Scaffolding is the frame work that is used by cells to adhere and grow. Scaffolds should have a high porosity and adequate pore size to facilitate cell attachment, provide nutrients to the cells, and give a direction for their proliferation [1]. The scaffold should degrade at the same rate that new tissue forms, thus providing structural support only until the new tissue can take over the mechanical load [2]. Mechanical properties, though important, are not the most pertinent attribute to consider when developing an implantable cellular scaffold [1]. The material's ability to support cellular proliferation and differentiation while at the same time offering a stable foundation for cell attachment, known as cell adhesion, is the paramount criterion [3]. Cell adhesion is dependent on far more than the type of material alone - the way in which the material is processed and its surface conditions also play a significant role in cellular adhesion [1]. Therefore, delineating viable processes for creating scaffolds is not exclusively centered on basic superficial attributes. Variables such as pore size and thread thickness are essential to optimize, but not at the expenditure of cell adhesion properties. The gold standard for cell adhesion is the emulation of the physiological condition to which cells naturally adhere to *in vivo*, the extra cellular matrix, or ECM. The ECM is comprised mainly of structural proteins, such as collagen and elastin, and acts as a support matrix for adjoining cells. The ECM by its nature is an elaborate system of collagen and elastin to which cells attach and proliferate. Bone tissue engineering using scaffolds involves the incorporation of viable cells, nutrients and a support structure to allow growth of new tissues in a significantly longer timeframe [4]. A current problem with creating bone tissue scaffolds is cell penetration to the center of a 3-dimensional scaffold. Recent research projects have only been able to achieve a cell penetration depth of 100-300 μm [8]. Cells receive nutrients by diffusion,

and therefore can not survive without close proximity to a nutrient source making it a challenge to produce large scaffolds more than 1 mm thick.

Bone tissue scaffolds would be beneficial in cases where a titanium or stainless steel plate cannot be used. An example where plates cannot be used is in cranial defects in children, which is often the result of a car accident, brain tumor removal, fall, or birth defect. Since the skulls of children are still growing, a fixed rigid plate would not grow while the brain and skull continue to grow. Fixed plates could sink into the skull causing further damage. Bone grafting from skull layers is not done in children because they have much thinner skulls than adults. The average skull thickness for adults is 7.0 mm while infants 12 months and younger have a skull thickness of only 2.5 ± 1.3 mm, and children 5-10 years old have an average skull thickness of only 4.6 ± 2.4 mm [5,6]. The thinnest skull thickness in children up to 10 years old is only 1.9 ± 2.4 mm [5]. Autologous bone from the rib or iliac crest can be used; however, there are several disadvantages that can be especially dangerous to children: these include increased blood loss, additional incisions, and infection to the donor site. Factors for special consideration in the repair of skull defects for children include the long term survival of the implant, cerebral protections, esthetic concerns, growing head, risk to donor site, morbidity due to length of the procedure, blood loss and resorption of the repair [7].

Proposal

The proposed solution is to create a custom bone scaffold that will be seeded with the patient's own bone cells or adipose-derived adult stem (ADAS) cells to create a patch of living bone tissue that can be implanted and grow with the child. To accurately create the bone piece, a Computed Tomography (CT) scan of the patient's skull is obtained and a 3-dimensional model is created using Mimics software (Materialise, Leuven, Belgium). Once the model is created, the simulation module in Mimics is used to extrapolate the missing skull fragment which is exported as a stl-file. Solidworks is used to project the 3D stl-file onto a 2D surface, which will provide the boundary for the scaffold mesh layer. A 2D mesh is created within the scaffold boundary according to the optimal pore size. The scaffold is fabricated in individual thin layers using in-house developed additive methods with polycaprolactone (PCL) and hydroxyapatite (HA). The thin scaffolds are then seeded with cells and allowed to grow and proliferate for several days. Seeding each individual layer before stacking ensures that the cells will have good coverage, and will be established at the center of the 3-dimensional scaffold. The seeded scaffolds are then stacked to achieve the 3-dimensional bone patch.

Material Selection

A powdered poly(caprolactone) (PCL) material manufactured by Dow Chemical Company (Danbury, CT) was used for the scaffold construction. PCL is a linear polyester that degrades into ε-hydroxy caproic acid in the body which is less toxic than lactic acid normally produced by other popular biodegradable polymers [11]. PCL is a highly crystalline and hydrophobic material which has an *in vivo* degradation time ranging from 1 to 2 years. Scaffolds made of PCL show a mass loss of 0.69% after 16 weeks compared with PGA which showed a mass loss of 64% during the same time frame, allowing the scaffold to sustain a higher mechanical load until regenerated bone can bear the loads [11]. Another advantage of PCL is that the pH of the surrounding tissue remains relatively constant. PCL has been extensively utilized in medical staples, stents, long term drug delivery systems and other medical devices.

PCL is much less expensive than other comparable materials with favorable material properties like a low melt point.

Hydroxyapatite was also utilized in powder form and was manufactured by Sigma-Aldrich (St. Louis, MO). For bone tissue engineering, blending PCL with hydroxyapatite (HA) holds great promise given that HA is the main mineral component of bone, and has been used as a bone filler and coating to promote bone ingrowth, especially in dental applications. Hydroxyapatite, like most ceramics, is brittle and difficult to fabricate into complex shapes, however combining with the more ductile PCL improves the toughness and strength of the overall scaffold. Many researchers have investigated effects of changing concentrations of polycaprolactone and hydroxyapatite. Rizzi *et al*. studied the effects of different polymer composites on cellular activity [9]. The combination of polycaprolactone and hydroxyapatite is believed to be a superior material for bone scaffolds than PCL alone [8, 2]. Marra *et. al* have also investigated PCL and HA blends for scaffold with rabbit bone marrow stromal cells and found both viable cells and formation of collagen up to 500 μm within the scaffold [8].

Manufacturing Methods

Extrusion, casting and laser melting were evaluated as possible candidates for the scaffold manufacturing process. Evaluation criteria for each process included the ability to maintain an optimal pore size for cells to proliferate, inclusion of micro surface properties for cell adhesion, incorporation of hydroxyapatite, and ability to maintain desired shape.

The target pore size was 300-400 μm which has been shown to promote osteoblast proliferation required for bone growth [10]. A rough surface finish added porosity at the microscopic level, providing the ideal condition for cell adhesion. Chloroform, used to dissolve PCL, is detrimental to cells and was fully evaporated before cell seeding.

Extrusion

A single screw extrusion system was designed and fabricated to extrude PCL (Figure 1). The 0.7" single screw has a three stage design: feeding, compressing and metering. The three different stages are temperature controlled, and can be adjusted along with the speed in order to optimize the extrusion of the PCL. The stainless steel nozzle moves in the z-direction while the build platform moves in the x-y direction giving the extruder the capability to fabricate a 3-dimensional part. In order to produce the scaffold, the powdered PCL was placed inside the feeder, heated and then the melted PCL was extruded onto a glass plate. The extruder has the capability to directly produce 3-dimensional curved scaffold layers. A stl-file of the bone patch was used to create a form controlling the curvature of the extruded PCL layer. The shape of the form was created through a Boolean operation and fabricated using a 3D printer from Z-Corp (Burlington, MA) as a low cost alternative.

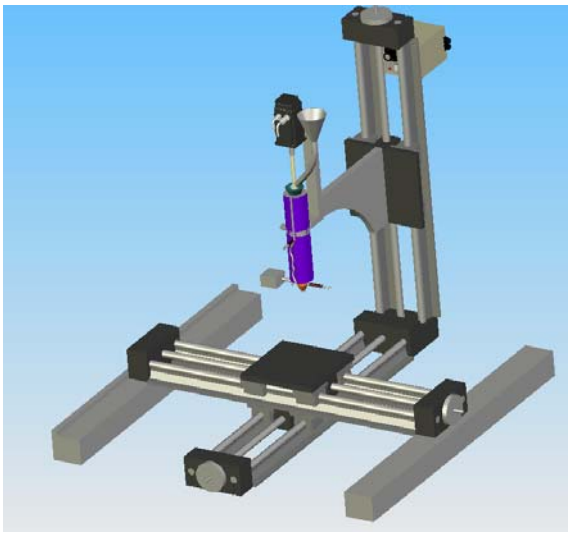
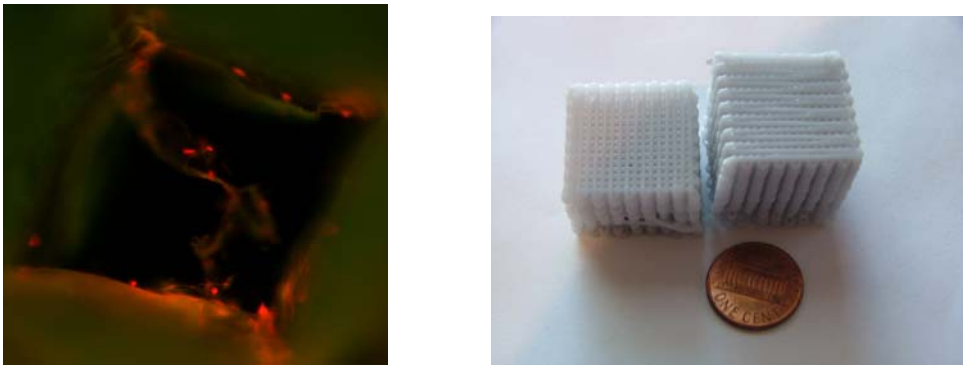


Figure 1: schematic of extruder

The extruded filaments had a smooth surface finish, and fluorescent staining after seeding for 24 hours with hADAS cells showed little cellular adhesion to the extruded scaffold (Figure 2). Experimentation involving the addition of hydroxyapatite (HA) with the PCL resulted in the heavier particles of HA settling to the bottom and clogging the nozzle, thus only pure PCL could be used. Although target pore size could be maintained, extruding small filament diameters was difficult due to the high pressure requirements at the nozzle. This method of fabrication is best used for filament diameters of 400 μm or larger.



a b

Figure 2: Extruded PCL scaffold, a. fluorescent staining of extruded scaffolds b. example of extruded scaffold

Casting

A second scaffold fabrication method was evaluated using casting of PCL into an open mold. The idea was to create large mesh structures that could be cut into the desired shape and curved using heat and a form. For the cell seeding experiments a mold with 3/8" diameter discs were fabricated from aluminum using a Computer Numerically Controlled (CNC) milling machine and a 1/16" ball end mill. PCL was dissolved in chloroform, then poured over the open mold and spread into the cavities. The PCL/chloroform mixture had a high viscosity, making it

difficult to fill the shallow cavities of approximately 1 mm. Additional chloroform was added to reduce the viscosity of the mixture, however, the evaporation of all the chloroform was a concern and caused extensive shrinkage. The resulting scaffolds had a smooth surface finish, and the pore sizes had significant variations due to the spreading of the PCL in the mold (Figure 3). However, because of the irregularities in pore size and the concern over the chloroform, this process was not considered for the HA addition evaluation.

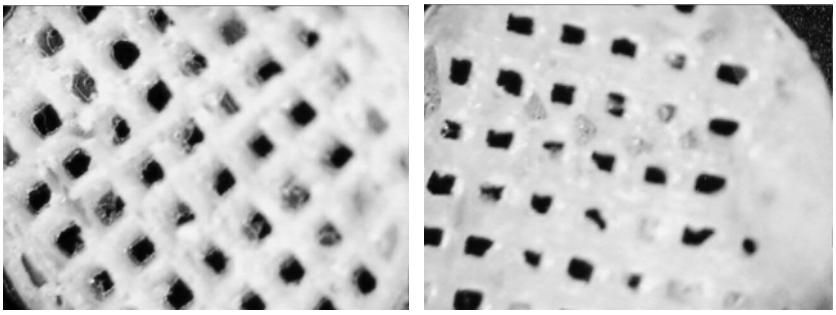


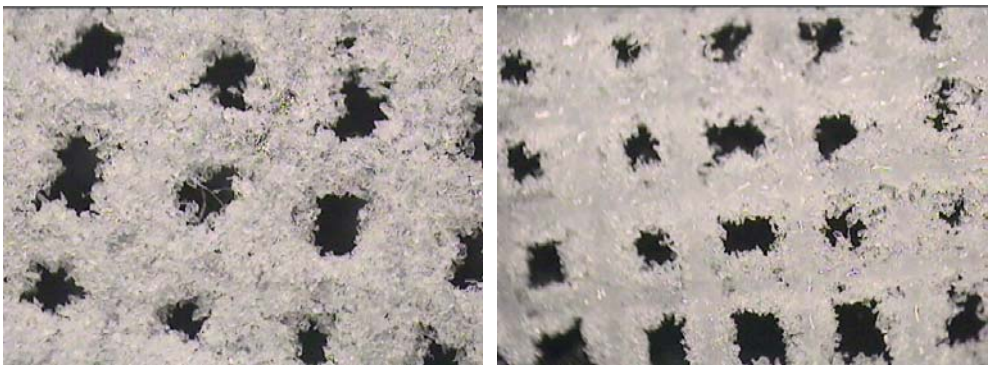
Figure 3: Cast PCL scaffold, 2x magnification

Laser Melting Method

The third method of producing PCL/HA scaffolds was to use a laser to melt the PCL in the desired pattern. Since the laser system on hand is a 2 dimensional system, the same process for creating curved layers, using heat and a form, that was used for the cast scaffolds was again employed. In order to melt the PCL powder, which has a low melt temperature, the parameters of the laser needed to be determined. A V-460 machine by Universal Laser Systems, Inc. (Scottsdale, AZ) with a 40 watt CO_2 laser and a 2" focal length was used to make the scaffolds. Solidworks (Concord, MA) was used to create a simple line pattern, and this was used to evaluate the laser parameters. Operational parameters on the laser system included speed, power and PPI (pixels per inch). Experiments were done with power at 15%, 20%, 25%, 30% and 35% of maximum and run at speeds of 60%, 70% and 100% of maximum. The criteria for determining the correct laser settings included sufficient melting capability for durability, ability to maintain the line pattern with an open pore structure, and avoidance of powder degradation due to burning. Power at 30%, speed at 70% and also the power at 20% and speed at 60% performed the best (Table 1). At these settings, there is a melt zone around the focal point of the laser, which causes the powder to be completely melted in the center, and sintered particles at the edges, giving the desired rough surface finish (Figure 4).

Speed	60%		70%		100%	
Power	Melt	Line pattern	Melt	Line pattern	Melt	Line pattern
15%	Too fragile	NA	NA	NA	NA	NA
20%	***Good***	***Open pores***	Too fragile	NA	NA	NA
25%	Good	Small pores	Too fragile	NA	NA	NA
30%	Good	Small pores	***Good***	***Open Pores***	Too fragile	NA
35%	Good	No pores	Good	No pores	Good	Small pores

Table 1: Results of laser speed and power parameters on the effecst of sufficient melting of powder to form a durable structure (Melt) and a line pattern that produced open pores (Line pattern). Best result at 20% power with 60% speed, and 30% power with 70% speed.



a b

Figure 4: PCL scaffolds produced on laser at 2x magnification. a. bottom of scaffold b. top of scaffold with visible increased melting

Measurement of the actual line thickness of the melted powder was not the same as the line thickness designed in Solidworks; however, a design of experiment (DOE) was conducted to ascertain the correlation between Solidworks line dimensions and actual line dimensions. Results showed that the pore size could be controlled effectively, and the minimum achievable line width is approximately 500 µm. Actual dimensions could be correlated to the Solidworks dimensions and these results were useful in subsequent scaffold designs.

Since this method of fabrication proved to be the most effective, additional evaluation was performed. Mechanical properties of the laser melted PCL scaffold were determined using an EnduraTec (Bose Corporation, Eden Prairie, MN) for several line thicknesses. The maximum tensile strength on samples was 0.875 MPa, slightly less than others' reported values of 1.1 MPa [8]. This decline is attributed to the laser only partially melting the powder PCL particles, effectively decreasing the cross sectional area.

Preliminary experimentation validated the assumptions regarding cellular adhesion and surface finish. Scaffolds were statically seeded with human Mesenchymal Stem Cells (hMSC) overnight in high concentration cellular media. The laser-melted scaffolds confirmed large quantities of cellular adhesion. From the research of the various production methods, laser-melting was chosen as the preferred method for creating cranial scaffolds.

Addition of Hydroxyapatite

In order to incorporate the HA, PCL is melted with the laser and the HA particles are captured within the melt. The powdered PCL and HA were thoroughly mixed at different percentages. A full factorial design of experiments using two levels of laser power, and four levels of HA concentration was conducted to determine the optimal laser parameters and tensile properties of the material. Material testing was done on an MTS Q-Test Machine (Eden Prairie, MN).

% HA	% Laser Power	Elastic Modulus MPa	Ultimate Yield Stress MPa
10	20	11.38 ± 3.04	0.128 ± 0.127
20	20	12.17 ± 0.69	0.085 ± .029
30	20	N/A	N/A
40	20	N/A	N/A
20	30	36.05 ± 7.45	0.414 ± 0.108
30	30	3.61 ± 0.39	0.011 ± 0.020
40	30	5.37 ± 2.16	0.018 ± 0.039

Table 2: Mechanical properties of test strips, sample size 10.

The laser power levels of 20% and 30% were tested with concentrations of HA at 10, 20, 30 and 40%. The 30 and 40% HA at 20% laser power was too brittle and could not be tested. Even at 30% laser power the higher concentration of HA samples were brittle and only a few of the ten samples could be tested. The 30% laser power and 20% HA proved to have the best mechanical properties, and were selected for the cell seeding evaluation.

Confirmation of the ability to maintain pore size of scaffolds while incorporating HA was done. The pore sizes were measured under the microscope as shown in Figure 5 below.

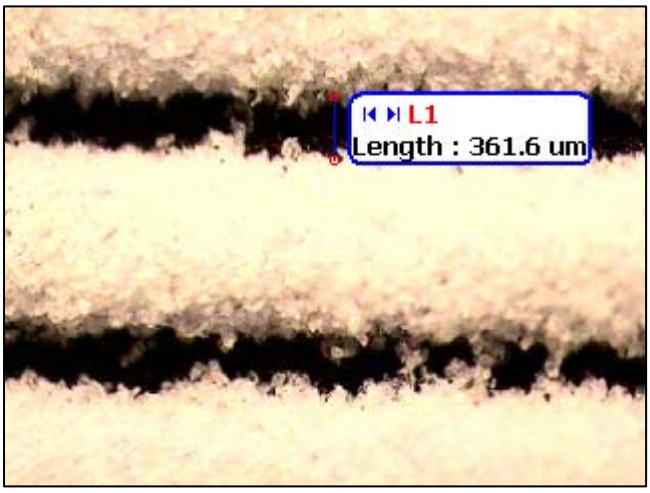


Figure 5: Measured Scaffold Line Space, 4x Magnification

Line spacing data and averages are shown in Table 2. This data indicates that the HA infused scaffolds have line spacing between 300-400 μm, with an average near 350 μm. When overlaid perpendicularly, this spacing will create a square-like pore with an edge to edge distance ideal for cell seeding.

Measured Spacing between PCL/ HA lines

Date	4/5/2007
Equipment:	National DC3-420T Digital Microscope
Software:	Motic Images 2000 Version 1.3
	**calibrated prior to use
Magnification:	4x

** All measurement values are in um

Measurement #	Scaffold 1	Scaffold 2	Scaffold 3	Scaffold 4	Scaffold 5	Scaffold 6	Scaffold 7	Scaffold 8	Scaffold 9	Scaffold 10
L1	271.4	316.6	372.9	351.1	384.4	340.8	316.6	429.5	430.0	418.7
L2	350.3	342.2	327.7	316.6	373.1	308.6	350.3	395.7	384.4	248.9
L3	316.4	180.8	373.1	372.9	384.2	331.0	327.7	395.5	373.6	259.9
L4	318.4	282.7	361.6	316.4	395.7	241.8	406.8	339.0	350.3	248.6
L5	350.3	361.8	305.3	361.6	372.9	328.5	339.0	384.2	429.5	282.7
L6	372.9	352.0	350.5	418.2	373.6	397.0	452.1	406.8	384.2	316.4
L7	305.1	395.5	327.7	395.7	373.1	331.0	429.4	440.7	271.2	260.1
L8	316.6	384.2	406.8	361.6	361.8	343.9	463.4	452.0	316.4	429.4
L9	282.7	395.7	418.2	384.2	271.2	328.5	406.9	350.5	294.0	282.7
L10	350.3	406.9	259.9	373.1	282.5	228.7	418.2	316.6	305.1	259.9
Averages	**323.4**	**341.8**	**350.4**	**365.1**	**357.3**	**318.0**	**391.0**	**391.1**	**353.9**	**300.7**
Standard Deviations	32.4	68.6	47.4	32.0	43.4	49.3	53.3	44.6	55.7	68.1

Overall Average	**349.3**	**um**
Average Standard Deviation	49.5	um

Table 2: Measured Scaffold Line Spaces

Cell Seeding

Human ADAS cell seeding was investigated with ½" diameter scaffolds. Individual scaffolds were created with a sewing ring in order for them to be stacked then sutured together. A thicker sewing ring was used in order to create spacing between the thinner scaffold mesh when the scaffolds where layered (Figure 6).

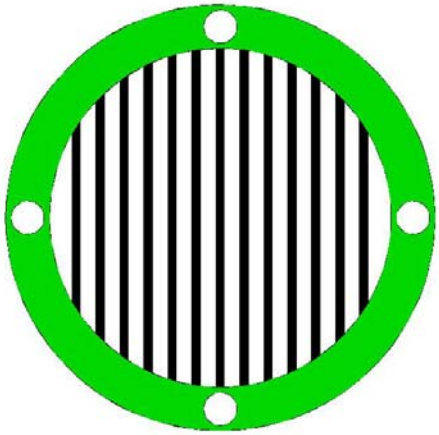


Figure 6: Schematic of scaffold with thicker sewing ring

To create the thicker sewing ring the laser power was increase to 50% of the maximum power (40 Watt $C0_2$ laser) on the sewing ring while still at only 30% of the maximum on the scaffold

line portions. Alternating the orientation of the lines resulted in a cross hatch mesh pattern of the final scaffold stack.

The individual scaffolds were seeded with hADAS cells at a density of 35,000 hADAS cells per scaffold. Growth media consisting of minimum essential medium Eagle, α-modified (α-MEM), 2mM L-glutamine, 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin was added to the scaffolds to allow for cell growth and proliferation. An AlamarBlue™ assay (AbD Serotec, Raleigh, NC) was performed at 72 hours post-seeding and confirmed that there were live viable cells on the individual scaffold layers before stacking. The AlamarBlue™ assay incorporates an oxidation-reduction dye that changes color in response to cellular proliferation. A solution was added to each well at a volume of 10% of the culture medium and incubated for 5 hours. The media was then sampled and the absorbance read at 570 and 600 nm using a Tecan GENios microplate reader (Tecan, Switzerland). Viability was determined by calculating the percent difference in reduction of Alamar Blue™ between experimental and control wells. The scaffolds were then stacked four high on a custom fixture then sutured together through the sewing holes and removed from the fixture. The stacked scaffolds were returned to the growth media and incubated (Figure 7).

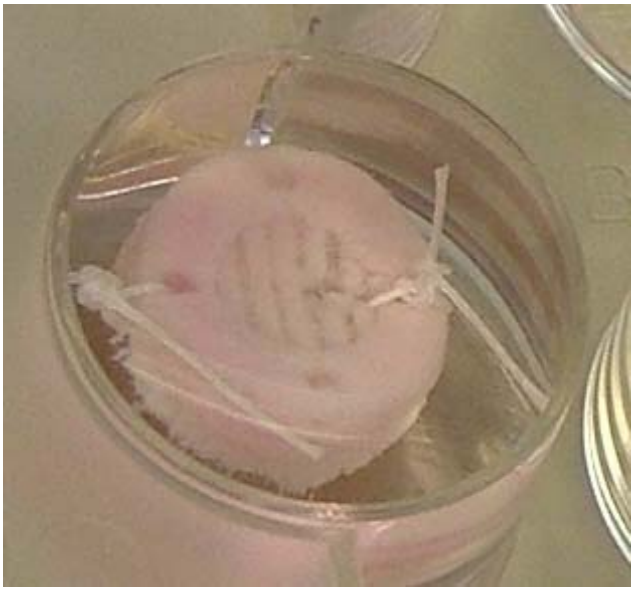


Figure 7: Stacked and sutured scaffold in plate well

The control well consisted of four seeded scaffolds which remained unstacked. At 24 hours, another AlamarBlue™ assay showed a decrease in the number of cells on the stacked scaffold, most likely due to some damage during the stacking procedure. However, the number of cells slowly began to increase again at both 48 and 72 hours (Figure 8). This confirms that the cells were able to survive and proliferate even after being stacked together. Future research will include placing the scaffolds in a flow bioreactor which will increase the nutrient flow to the center cells.

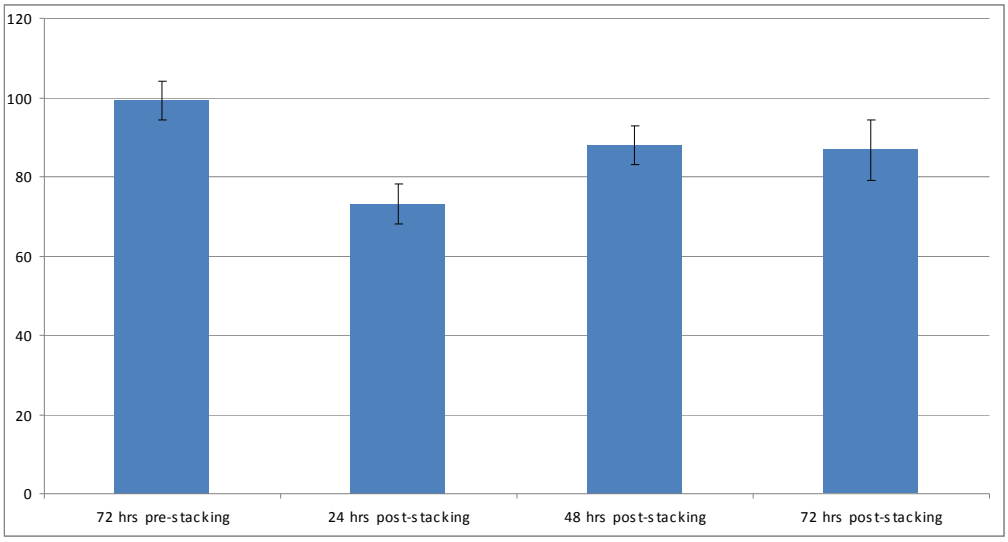


Figure 8: AlamarBlue assay results for ½" diameter scaffolds, pre-stacking, then post-staking and sutured together.

Scaffold shaping

The method for creating the scaffolds necessary for a custom bone patch was also investigated. A CT scan of a patient's skull with a defect was acquired and Mimics (Materialise, Belgium) was used to create a 3D model of the skull (Figure 9).

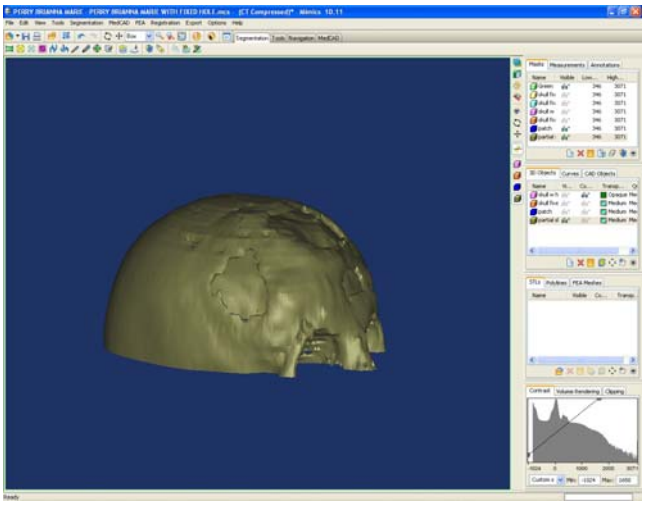


Figure 9: screen capture of child's skull showing defects in Mimics software

The simulation module in Mimics was used to extrapolate the missing bone fragment, which could then be exported to Solidworks as a stl-file. Solidworks was used to create a model of the curved bone fragment. The model was then produced on a Dimensions SST rapid prototyping machine (Stratasys, Inc. Eden Prairie, MN). The model was used as the bone fragment mold for scaffold shaping. The boundaries of the stl-file were used to create the boundary of the scaffold. Using Solidworks the 3-dimensional bone fragment model was projected onto a 2-dimensional plane to create the thin scaffold layers. The scaffold mesh line pattern was then created to obtain the appropriate pore size of 300-400 μm. Alternating orientation of the scaffold lines was done

to create a mesh pattern upon layering. The final 3-dimensional scaffold shape was obtained by heating the bone fragment mold and layering the scaffolds on top, resulting in the scaffolds taking the curved shape of the form (Figure 10).

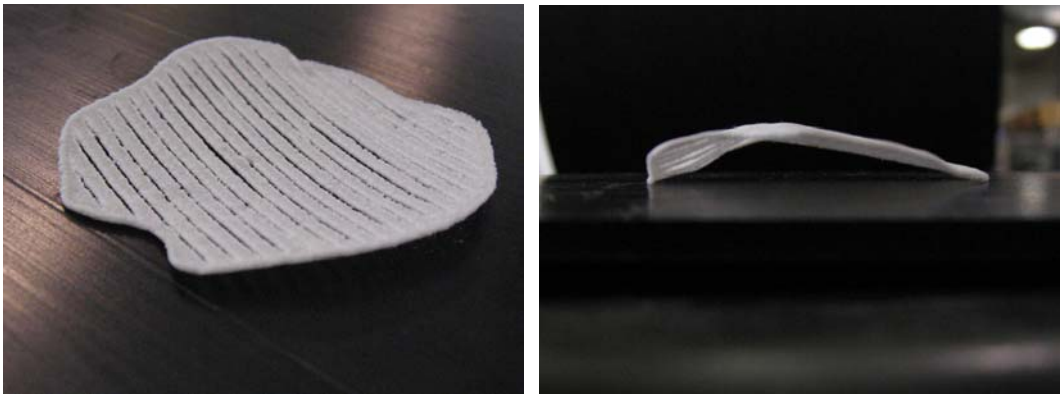


Figure 10: Scaffold layer with curved skull shape

In the future, these curved layers can be seeded and stacked using the same procedure as the 3/8" diameter discs to create a custom bone patch.

Conclusions

Several fabrication methods were evaluated for production of scaffold layers for bone tissue engineering. Extrusion led to production of a 3-dimensional shaped scaffold, while the casting and laser melting required a heated form. Extrusion was limited to pure PCL scaffolds with filament diameters of greater than 400 μm. Casting with a PCL/chloroform mixture proved to be a viable method, however, pore size was difficult to control and chloroform evaporation was a concern. Laser melting was able to achieve optimal size as well as produce a surface that enhanced cell adhesion. The individual scaffolds were seeded and stacked with limited damage to hADAS cells on the scaffolds, giving even cell coverage within the 3-dimensional scaffolds. Custom scaffold were created from CT scans to match a 3-dimensional bone defect. Although this research focused on infant skull defects, the process can be applied to any bone defect.

Future Research

Future research will included seeding the shaped scaffold, using a flow perfusion bioreactor, and including flow channels to optimize nutrient flow. Only 2 dimensional disc shaped scaffolds were seeded with hADAS cells for the initial evaluations, future research will include seeding the curved 3-dimensional scaffolds. Static cell seeding was done initially; however, using a flow bioreactor will increase nutrient supply to the center of the scaffold stack. The scaffolds created for the initial experiments did not include any flow channels through the scaffolds. For larger bone patches, cells would need close proximity to a nutrient source and flow channels could easily be incorporated into the Solidworks mesh design. Future research plans also include seeding the scaffolds with hADAS cells for longer time periods enabling the cells to form bony constructs. Tensile testing to determine the mechanical properties of the seeded scaffolds is also planned. The research already conducted has laid the ground work for additional research into custom bone patches. The method of seeding thin individual scaffolds layers, then stacking and suturing them together will be further investigated with the ultimate

goal of achieving a custom bone patch that is grown *in vitro*, implanted in a single surgery and can grow with the child.

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