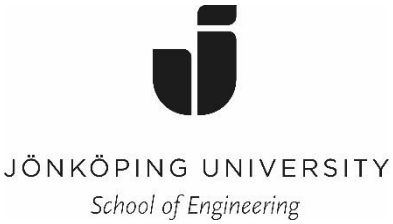

Design, 3D Bioprinting, and Testing of Otic Prosthesis

PAPER WITHIN *Product Development and Materials Engineering*

AUTHOR: *Dan Rogozea*

TUTOR: *Nicanor I. Moldovan, PhD*

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The author takes full responsibility for opinions, conclusions and findings presented.

Examiner: Roland Stolt

Supervisor: Morteza Poorkiany

Scope: 30 credits (second cycle)

Date: August 17, 2018

Postadress:	Besöksadress:	Telefon:
Box 1026	Gjuterigatan 5	036-10 10 00 (vx)
551 11 Jönköping		

Abstract

The middle ear is a complex organ with multiple functions. It is prone to accidental, genetic, excessive noise exposure, or age-related damage. Its main role is to convey and amplify the mechanical vibrations of the tympanic membrane to the acoustic nerve through three connected small bones, the ossicles; malleus, incus, and stapes. When severely damaged, the most used solution is currently a partial or total ossicle replacement with inorganic titanium prostheses, which are not anatomically similar. However, 3D models derived from micro-CT scans of human ossicular chains are freely available for research and educational purposes in high resolution 3D files. Therefore, these files were scaled to anatomical size and used to print partial models of the malleus and incus using an extrusion contact method using a bioprinter and OsteoInk, a calcium phosphate-based paste sold by the bioprinting company regenHU. The use of this biologically analogous material to 3D print anatomically sized and shaped ossicles is novel. The process and settings for bioprinting the malleus and incus were devised and tested for repeatability. OsteoInk was found suitable to form hard bone-like objects after printing and curing. However, for this process to be successful with OsteoInk, the models required a flat base; the first .560 mm of the virtual model were not printed. A support structure is required for creating complete ossicles, but the hydrogel and polymer structures attempted were not deemed feasible. The support structure could be created by combining the OsteoInk with other biomaterials, or by fibers printed through Melt Electrospinning Writing. The workflow devised in this project is applicable to other bioprinters, and to thus to further the research in the field of bioprinting.

Summary

The main purpose of this project was to create a process for bioprinting human ossicles prothesis with a biocompatible bone-like material (OsteoInk). The combination of anatomic models, this material, and 3D bioprinting is novel. This project investigated the feasibility and detailed the process of bioprinting ossicular implants. The 3D virtual models were analyzed and found to be anatomically correct as well as modifiable for individualized size and shape of ossicular prostheses.

Before starting this project, it was unknown if the 3D virtual models could be used, modified, or even if the material and bioprinter would be suitable for creating ossicle prostheses. The need and utility of a support structure for the ossicle prints was also investigated. The 3D printing methods (extrusion, inkjet, melt electrospinning, etc.) for the printing of ossicles and/or support structure were studied. The repeatability of the process was determined.

This project started with the comparison of the ossicle models to anatomical measurements. The models were then scaled to the appropriate size and further modified in CAD. The ossicle computer models could be virtually modified, were found useable for printing with OsteoInk in a bioprinter, and the process created for this was proven repeatable. The computer code required to print these ossicle models was found to be transferable to other bioprinters.

The required settings and other specifics of 3D bioprinting were first investigated with a surrogate bioink (Nivea crème) and the findings were afterwards applied to OsteoInk. It was found that OsteoInk cannot support its own weight and thus cannot be printed without a proper support structure, unless the models were cut down until a flat and supportive layer was determined. This procedure allowed for the printing of ossicle models, but these printed ossicles were not dimensionally complete. Typical scaffolding materials used in bioprinting such as polymers through extrusion, or hydrogels through inkjet microvalve deposition, were also attempted. These methods and materials were found to be incompatible with the OsteoInk and the available bioprinter. The novel method of Melt Electrospinning Writing for producing a support material as a polymeric fibrillar mesh was also attempted and showed promise, but further investigation is required. The repeatability of printing partial ossicles was investigated and met the proposed goal set in this project.

The stapes was found to not be printable with the same process used for the malleus and incus, due the very small size. Thus, further investigation into bioprinting technologies is required to print the whole ossicle chain. Further investigation of different biomaterial or possibly combinations of OsteoInk with other biomaterials is required. Advancements in OsteoInk-based blends could result in printing without a support structure as well as more biologically compatible prothesis. The process detailed in this report will be applied to further the research in this field, and to its medical applications.

Keywords

3D Bioprinting

Additive Manufacturing

Biocompatible Material

Bioink

Ossicles

Implant

Tissue Engineering

Contents

1 Introduction

1.1 Background

1.1.1 Sound Perception, Generation, and Processing in the Animal Kingdom

Sound has played a crucial role in development of many species including humans by providing notice and communication with other co-specimens, and warning of possible dangers nearby. The development of sound processing and noise differentiation has allowed early humans to learn to distinguish between threats or harmless (even pleasant, as in music) noise. While humans may not have the best hearing ability compared to other species, the processing of sound has helped humans thrive in both nature and advanced through the formation of social groups.

This has been made possible by the co-evolution of sound generation in the mouth, receiving by the ear and processing in the brain. The evolutionary advances of the sound generation organs and the oral cavity have played a crucial role in the development of human society as it has allowed efficient communication. Humans are social creatures and have been able to co-integrate through their communications. In these groups, humans have been able to rapidly advance by sharing knowledge through speech. Before the invention of writing, oral traditions have played the most significant role in advancement of humans. Sound and the ability to communicate have therefore been a crucial developmental advantage.

1.1.2 Speech and Hearing in Human Society

Throughout human history, hearing has provided protection from threats (sounds), communication (language), and as a method of passing on compounded knowledge (instruction). Language is among the defining functions of humans and one of the most powerful communication tools available. Before the invention of written communication, most of information was transferred between people and generations orally. The alternative to speech, drawing, was crude and highly susceptible to interpretation. Traditions, knowledge, community integration, and entertainment has been mostly passed down through speech for the majority of human history. Written communications are a relatively recent advancement in human history and a development derived from speech. The integration of humans in groups has also highly benefitted from oral communications and has been the precursor to the large societies and cities of modern times. In contemporary times humans still segregate themselves in societies based on oral communications and languages. Hearing has therefore played a large role in the formation and maintenance of human societies.

1.1.3 Medical Burden of Hearing Loss

Due to a combination of several key genetic or environmental factors, the frequency of hearing disabilities continues to steadily increase in the contemporary world. According to the World Health Organization, over five percent of the world's population of 466 million people have debilitating hearing loss. That number is expected to rise to 10% or over 900 million people by 2050. Approximately one third of all humans over 65 years of age face disabling hearing loss. [1]

While hearing loss does not represent a major setback during contemporary times and societal integration is no longer strictly dependent on the ability hear, it may compromise the quality of life. If the ability to engage in society through media or to easily communicate is diminished the quality of life suffers. Advancements in the ability to restore hearing have been prevalent in previous decades, but are still not occurring at the pace required to effectively treat all cases and help the large population affected. While it is easier than ever to communicate without the ability

to hear in contemporary society and most dangers in life do not rely on hearing to be detected, the need for hearing restoration is still as important as ever.

1.1.3 Origins of Hearing Loss

Hearing loss can be largely broken down into the categories of genetic and induced:

a. Genetic Hearing Loss

Genetic hearing loss is one of the most common birth defects and affects as many as three of every 1,000 babies born. [2] Genetic hearing loss commonly occurs after birth as well. Otosclerosis is an example of a genetic disorder which may cause hearing loss around the ages of 10-30. This is the general term for irregular growth or reformation of inner ear bone osteo cells. [3]

b. Induced Hearing Loss

Induced hearing loss is mainly due to the constant or occasional (if intense) environmental exposure to modern lifestyles. Sources include but are not limited to power tools, motor vehicles and amplified music. Induced hearing loss is occurring at more frequently rates in both children and adults. Occupational hearing loss is also increasing at an alarming rate due to lack of hearing tests, inadequate use of hearing protection, and poor awareness of dangerous noise levels. [4].

1.1.5 Structure of the Middle Ear

The middle ear bones, the malleus, incus, and stapes, or ossicles, (Figure 1) form a chain which mechanically transmit the vibrations of the tympanic membrane to the cochlea. The cochlea is filled with fluid and transmits the vibrations to the cilia (the hair cells of the inner ear) which are later interpreted as sounds to the brain. The ossicles are an essential part of hearing system while being fragile and the most exposed part of the chain.

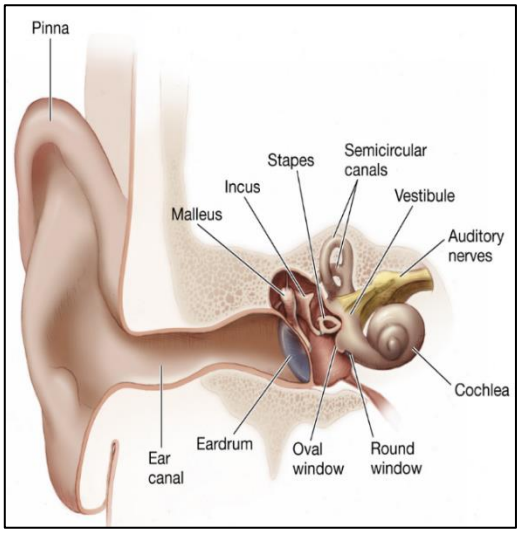

Figure 1: Anatomy of the middle year. Shows the position of the incus, malleus, and stapes in the middle ear. [5]

1.1.6 Mechanisms of Hearing Loss.

Physiologically there are three types of hearing loss: conductive, sensorineural, and mixed (conductive and sensorineural).

a. Conductive Hearing Loss

Conductive hearing loss occurs due to damage or irregular growth of the ossicles, and occurs mostly from head trauma or otosclerosis. This form of damage results in fractured, immobile, or an altered position of the three middle ear bones (ossicles); the malleus, incus, and stapes. The primary objective of the middle ear is to transmit external sound vibrations to the tympanic membrane. If a portion of the sound transmission chain is broken or otherwise nonfunctional, hearing loss occurs. The extent of this hearing loss depends on the extent of the damage to the ossicles.

b. Sensorineural Hearing Loss

Sensorineural hearing loss is caused by nerve damage in the sensory pathway and particularly to the cochlear hair cells. This type of hearing loss can occur at birth or is acquired later in life. The most common form of this type of hearing loss is of the acquired type due to constant exposure to loud noise. Replacement ossicles will not solve any nerve damage and replacement ossicles will not be applied to sensorineural problems.

c. Mixed Hearing Loss

Mixed hearing loss (conductive and sensorineural) is the combination of damage to the ossicles and the nerves which are responsible for the transmitting sound. Replacement ossicles might partially solve mixed conductive and sensorineural hearing loss.

1.1.7 Current Approaches to Conductive Hearing Restoration

a. Surgical Repair

Surgical repair is often used to directly or partially solve ossicle related injuries. If the ossicle is required to be repositioned, the surgeon will be able to accomplish that feat. Removing material, due to otosclerosis, or adding material such as hydroxyapatite cement to repair fractured ossicles, are common surgeries. The use and success of these surgeries depends on the skill and practice of the surgeon. [6]

b. Ossicle Prostheses

The most widely utilized contemporary method of restoring conductive hearing is the total or partial replacement of the ossicles with a biocompatible titanium implant [7] or using ossicles donated from other humans. [8] The most common surgical intervention involves titanium prothesis implants of various shapes and sizes for partial or total ossicle replacement. [9] Examples of the implants can be seen in Figure 2. Surgeons must determine the best options based on the size and shape required as well as if all or just a portion of the ossicles will be replaced. The total number of variations in size and shape of ossicle replacement prothesis are limited to a few standards determined by the manufacturers. Limitations of current manufacturing processes also further limit choices of titanium implants and restrict the possible shapes of such implants.

Figure 2: Example of available middle ear titanium implants. Titanium implants can be semi-custom (Configure to order). [10]

Several problems are encountered with the current titanium prosthesis models, most notable being the success rates. Air to bone gap measurement are used to determine the success of the implants. The lower the gap the closer the ossicles and therefore the higher the vibration transmission. Published success rates consisting of low bone to air gaps have been between 57% and 77%. [9] Healing time has been reported at an average of 24 months. [9] While ossicles size can vary extensively within individuals in similar genetic populations, [11] titanium prostheses are limited in their customization and size availability.

Research reveals that the best implant should "from a surgical standpoint, require easy manipulation, reduce surgeries to partial or total variants, and be constructed of stable, biocompatible material. From an acoustic standpoint, a prosthesis should weigh 10 to 40 mg, provide proper tension between the tympanic membrane (TM) and stapes, form less than a 30-degree angle with the TM, and accommodate the malleus." [12] This study though was completed before the invention of biologically analogous materials. Titanium was the preferred material at the time. 3D printing or other forms of additive manufacturing were also not considered at the time of this study. The compromises expressed by the author hereby can be mitigated and an implant similar to natural ossicles could be made in both shape, size, and acoustic performance through the use of biocompatible materials and 3D bioprinting.

1.1.8 Additive Manufacturing ('3D Printing') Technologies

Additive manufacturing provides the ability to make custom sized and custom shaped implants. Figure 3 shows a proposed design for an implant made by 3D printing (additive manufacturing) techniques to solve the lack of sizing options in ossicle prothesis. [13] While this might be a more effective solution in comparison to the commercially available titanium prosthesis, the shape is clearly not biologically equivalent. The material is also not biocompatible and does not integrate well into biological systems.

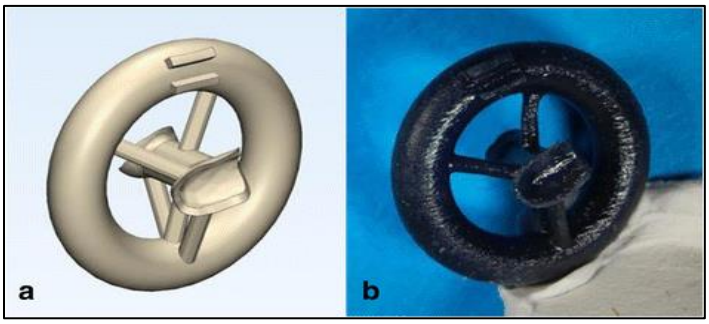

Figure 3: Proposed 3D printed polymer based individualized implant. CAD model on the left (a) and printed part on the right (b) [13]

Due to the large variations in dimensions and shapes of each ossicle between individuals, i.e. 'personalized,' the manufacturing of prostheses should be optimized for each patient. Accurate 3D models of the malleus, incus, and stapes have been previously created from CT scans [14]. These models could be modified for patient-specific anatomy using high-resolution 3D image reconstitution of histological sections and by computer-aided design (CAD) [15] to be individualized. Models could also be directly obtained from micro computed tomography (micro-CT) scan data for each patient. [16] However, this process is time consuming and expensive. The best compromise seems to be to measure the patient's size and shape and edit accordingly to make ossicles which fit. The manufacturing of these individualized medical implants is suitable for additive manufacturing due to the high cost of the parts (if compared to other manufacturing methods) and due to the variable dimensions.

1.1.9 3D Bioprinting

Bioprinting, creating 3D structures from cells and/or biomaterials through individual layer deposition, is emerging as a powerful new technology of tissue engineering. [20] Techniques and technologies pioneered for the use of 3D printing of materials such as polymers and metals can be applied to biological or biologically compatible materials. Bioprinted implants could replace traditional implants and improve outcomes for all patients through more biologically compatible or even tissue engineered implants.

At the current time there is no process for the creation of ossicles through bioprinting with biomaterials and/or cells. Several methods are available for creating models and materials that mimic bone. The challenge in creating bone like ossicle implants is utilizing the best combination

of the possible methods and materials. Resolution of the print is also a major challenge due to the small size of the ossicles. To our best knowledge, there has not previously been an attempt to combine all aspects required to print ossicles prothesis in a biological similar shape and material.

1.2 Purpose and Research Questions

1.2.1 Purpose

This thesis will investigate if the models of the ossicle bones can be altered in CAD or similar software. As medical imagining faces numerous limitations discussed in section 1.4.1, it will be investigated if the available 3D models are sufficient for the creation of the anatomically correct prothesis models as well as if these available 3D models can be edited for patient specific customization. It will also investigate if the ossicle models can be printed by additive manufacturing techniques using a commercially available 3D bioprinter.

1.3 Research Questions

1.3.1 Design

The process of 3D printing middle ear ossicles with biomaterials has not been previously investigated in detail, therefore we must prepare and/or modify as needed to create printable files compatible with available bioprinters.

Question 1: Are the available ossicle models appropriately sized and comparable to natural human ossicles in shape and features and could these models be modified?

The initial part of the project will determine if the 3D models are sufficiently detailed to be used to print and if these models can be printed in the required size.

Question 2: Are the available bioprinting methods adequate for printing ossicles?

As the ossicle bones are some of the smallest in the human body and the material properties of the printing biomaterial are unknown, the 3D printing method (extrusion, droplet, etc.) and materials for this application are novel and will require more research.

Question 3: Will a scaffold or supporting structure be required during the printing of the ossicles?

The need and the optimal solution for a supporting scaffold is unknown and will need to be assessed starting with its design and up to the practical implementation.

Question 4: Which materials and methods are appropriate and practical for such a structure?

The materials and process required to create a scaffold for the ossicles will be new and unexplored.

Question 5: Can the process and the product of the printed ossicles meet the expected quality level, and could this process be repeated at the same quality level?

The prothesis cannot be useful if it is not able to reliability created. The quality must be acceptable and the ability to meet this quality must be repeatable.

Question 6: Can this process be transferred to other bioprinters?

The process is preferred to be useable on other commercially available bioprinters. The procedure of how this could be accomplished will need to be determined.

1.4 Delimitations

1.4.1 3D Model Creation

This thesis will not cover the original creation of the virtual (computer) ossicle models. Medical imaging faces numerous ethical, financial, and practical challenges, therefore obtaining CT scans from patients and processing these into 3D models is beyond the scope of this thesis. This thesis is also not related to 3D imaging or 3D image processing, as such it will not be covered. 3D models derived from micro-CT scans of human ossicular chains are freely available for research and educational purposes in high-resolution. [17]

1.4.2 Material Properties

The physical and mechanical properties of the printed objects will also not be investigated in this study. The process developed for bioprinting ossicles could be further implemented with similar materials but finding the ideal material will require more research. However, this study will be broad and applicable to most other cell-free materials. Future areas for research include using live bone cells, or live cells in a mixture with a matrix and will add complexity to the material properties.

1.4.3 Constructs Testing

Neither *in vitro* (mechanical) nor *in vivo* (functional) testing of the prosthesis will be conducted during this study. The actual implantation of such prosthesis is beyond the scope of this study. The requirements of implantation surgery, such as altering the shape of the ossicle to optimize handling during surgery, will also not be studied as that is in the field of medicine and surgery. The resources for a surgical study are not available and the prothesis will not be ready to implant at the end of this project. The bioprinting process created in this thesis will be applicable in future applications for surgically optimized ossicles.

1.5 Outline

This report will continue with a theoretical background of relevant topics in bioprinting including technologies and biomaterials. The Methods section describes how the project was conducted and the different phases completed. The Findings section reports the results of the Methods sections. The Discussion section concludes the project and designates future research.

2 Theoretical Background

2.1 3D Printing or Additive Manufacturing

Additive Manufacturing has been a disruptive technology in almost all industries; the medical and biotechnology industries have not been an exception. The basic concept of 3D Printing, often called 'additive manufacturing', is to deploy a semi-solid material printed in a layer-by-layer mode to create a shape. Traditional methods of manufacturing are based on the subtraction of material, such as the manufacturing of metal prostheses through machining. In our case, the major advantage to using additive manufacturing for creating implantable ossicle protheses is the ability to customize the final product according to the requirements of individual patients. This comes from improvements in medical imaging, computer modeling, and 3D bioprinting.

The basic process of generating 3D objects that can be printed involves image acquisition, segmentation and editing, mesh generation, conversion to STL files, sending the data to the printer, and then determining the parameters required to print. Models are formatted in CAD software and sent to the printer in STL surface mesh files. The models are then sliced to create the layers which will be printed. Figure 4 shows the process of taking a 3D model, conversion of the model to a mesh, and an individual layer.

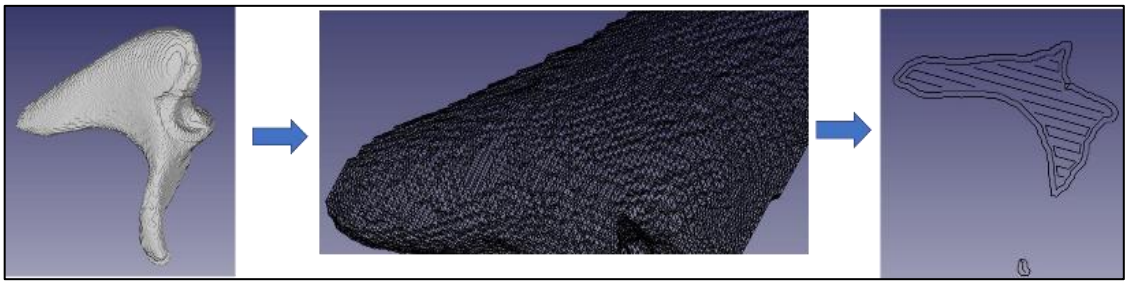

Figure 4: Example of 3D model (ossicle) processing. The initial model is processed into a mesh which is then transformed into specific printer path with infill. The rightmost frame shows a single layer that bioprinter will create.

2.2 Medical Imaging Technologies

The first step in the process of creating customized implants is determining the size and shape required. Starting from an anatomically correct image is crucial, as this serves as the base design for editing. Initial research around the creation of ossicle models came out of the need for teaching tools. Advancements such as hand created finite element models were created, but the idea of using these models for 3D printing was not considered at the time. [18] 3D imaging was later utilized to create more realistic models with increasing levels of resolution through continued research. Methods which used x-ray, micro-CT, or histological sectioning technologies were used, but historically the small details were not properly rendered due to the low scanning resolution. [17]

The small size of the ossicle bones requires high resolution scans, but also makes obtaining the medical images more difficult. Models used in this project came from medical imaging research completed at the University of Antwerp. [17] The models of the ossicles were derived from extracted bone samples and utilized a custom built micro-CT scanner able to feature recognition up to 2 μm. [17] The bone models were scanned in 360° and 1000 images were taken of each model. The images were then reconstructed into 3D image files or coordinate files using custom software. The images were further processed through manual segmentation. [17] This resulted in models of each bone which were individually released rather than a single file of the whole connected chain. Computer models can be saved as full 3D images or as a series of X,Y,Z coordinates. The most commonly used files for 3D printing are STL, which is an outer shell of a 3D structure. The models used in this project were in the STL format due to the requirements of being edited in CAD software.

2.3 Bioprinting

3D printing is the creation of an object through the addition of material layer by layer. Bioprinting is 3D printing with biologically compatible materials and/or cells. Bioprinting is broadly defined as "the use of material transfer processes for patterning and assembling biologically relevant materials (molecules, cells, tissues, and biodegradable biomaterials) with a prescribed organization to accomplish one or more biological functions." [19]

Bioprinting can be further broken down into two categories based on the use of a scaffold: 'scaffold-dependent' and 'scaffold-free' bioprinting. Scaffolds can be created out of biologically compatible materials which don't elicit negative reactions in the biological system (biomaterials) to support the main print in scaffold-dependent applications, or self-supported cell-only based constructs in scaffold free. The latter can be further described as arranging cells into desired structures using 3D printers, hybrid biologically compatible material and cell structures, and cell maturation techniques.

Traditional fabrication methods, removing material from a larger starting block of the material, are highly impractical when dealing with biomaterials and cells. These methods cannot be used to remove cells from a larger mass of cells and are not efficient when making small biological models using only biomaterials. 3D bioprinting is a great solution to this issue.

2.4 Bioprinting Techniques

Several methods have been developed for 3D printing of polymers or other materials. [20] Ideas and concepts from these methods can also be used for printing of biological or biologically compatible materials by modifying the process or using different printing materials. Examples of methods which have been successfully applied to bioprinting include extrusion, inkjet printing, valve-based printing, light and laser processing, or melt electrospinning. The combination of these techniques can also be used.

The computerized techniques for model processing, modification, or slicing can be directly applied to bioprinting. The placement of the material on the substrate is controlled by servos and translated from X, Y, and Z coordinates given to the machine in numerical control programming language (G-code). Standard 3D printing software, such as the open source OctoPrint [21] or Slic3r [22] programs, can be used to place, slice, convert the model to G-Code, and to control the 3D printer. More elaborate and specialized software packages, such as those specialized for tissue printing, are also available and can simplify the 3D bioprinting process.

The methods used or considered in the project: extrusion, micro valve, scaffold free, and melt electrospinning writing, will be described in this section.

2.4.1 Extrusion

The most common method for additive manufacturing is fused deposition printing. This involves the melting and extrusion of a polymer-based materials and then depositing them into layers. Due to the heat and extrusion, this process is not feasible for most biomaterials. Cells and other fragile compounds must be handled more gently. Extrusion is a contact method where the printed material makes a direct contact with the substrate when printed. Therefore, a simple process involving air pressure or even minimal mechanical force without heat or extrusion is the best choice.

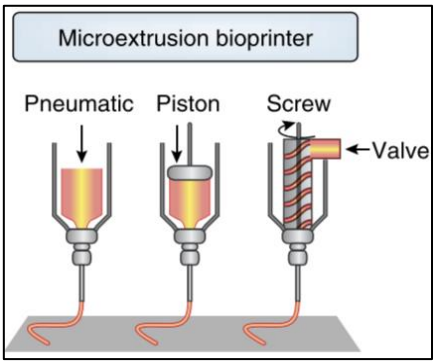

Figure 5: Example of 3D bioprinting with extrusion. Three types of extrusion methods are shown. Pneumatic and piston are the most common. [20]

Materials which contain cells are fragile and do not respond to shock well. High accuracy pressure regulators, air or mechanical, can be combined with off the shelf syringe cartridges and nozzles for extrusion. Figure 5 displays the types of dispensing mechanisms possible. The cartridge and nozzle choice are extensive and commercially available. The combination of pressure settings and dispersion units can be used to print large or small constructs of almost any material and at practically any speed depending on the impact resistance of the printed biomaterial.

2.4.2 Microvalve or Inkjet Printing

If the printing material is not desired to be in contact with the substrate material, microvalve (inkjet) 3D bioprinting can be used. The same principles as in inkjet printers are applied in this technique: drops are deposited on the substrate based on a computer program. Two main types of inkjet technologies can be used: piezoelectric and thermal. In piezoelectric based techniques, an actuator controls the valve which releases the printing material. A short current pulse is applied to the piezoelectric element which causes a change in the shape of the reservoir and the material to be released. [23] While not a requirement, pressurizing the material chamber results in more precise material control and better results. Thermal inkjet systems consist of a heating unit and a chamber with small orifices of 30-200μm. The electric current is applied to the heater which heats up and ultimately causes an air bubble to form which provides enough pressure to push the material through the orifices. [24] Figure 6 shows the two main types of inkjet printer heads.

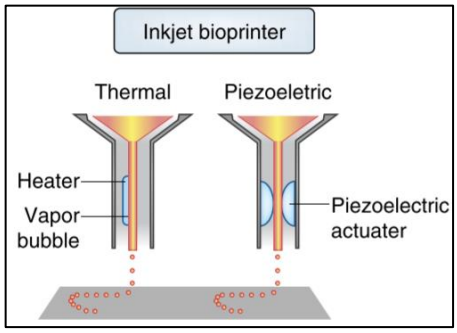

Figure 6: Example of a 3D bioprinting with inkjet method. Thermal and piezoelectric. [20]

The two methods provide similar benefits and drawback. The main drawback is the inability to use highly viscous fluids. Only low viscosity liquids will be able to be printed with this method as others will clog the print head. The other major drawback is the high mechanical stress placed on cells or printable materials. Various forces such as hydrostatic and shear are placed on cells. The impact of being placed on the substrate is also a drawback. The major advantage is repeatability and fine control of the print. Resolution though is relatively low in practice due to the viscous nature of the material and the orifice nozzle sizes. [26]

This method can be used to print support scaffolds, easily repeatable structures, or in a combination with other bioprinting methods to add cells or build supporting layers. It is especially effective with hydrogels due to their low viscosity.

2.4.3 Scaffold-Free Bioprinting

This method is a specialized robotic assembly system that can assemble cells in a structure without the need for any support material. Cell spheroids, cultured cell aggregates in sphere formations which contain tens thousands of cells and are around 500 μm in diameter, are robotically assembled in a needle array made of stainless-steel needles with diameters of 100-200 μm, named 'Kenzan' by their Japanese inventor. [25, 26] . The assembly can be arranged through a computer program and the number of spheroids constrained by the shape of the construct and the dimensions needle array. The needle array is illustrated in Figure 7. Once the desired structure is created, the 3D spheroid construct is cultured in a bioreactor to allow spheroids fusion by cell rearrangement and reorganization. Tissue function and strength is achieved in this stage. [25] The main advantage of this technology is the ability to create cell-based prints without the need for support structures. The main disadvantages are the low resolution constricted by the needle array, and the need for spheroid cells. The process is also time consuming and much more expensive than other methods.

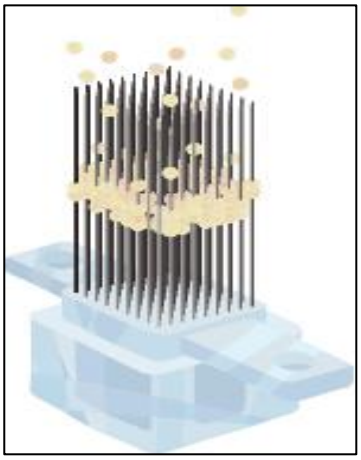

Figure 7: Needle block ('Kenzan') for scaffold free biofabrication: A robotic arm controls the placement of cell spheroid on this microneedle array [25]

2.5.3 Melt Electrospinning Writing

Melt electrospinning writing (MESW) is a specialized form of electrospinning. In traditional electrospinning methods, a polymer is dissolved in a solvent and then drawn out on an electrified (grounded) glass surface. [27] A pressurized cartridge helps eject the printable material. This creates very thin drawn polymer fibers, but it is hard to control the 3D structure. Melt electrospinning uses low melting point polymers, especially poly (ε -caprolactone) or PCL, and a heater to melt rather than dissolve the polymer. [28] This eliminates the issues with having to use a solvent, which is incompatible with cells. The MESW system includes all the typical electrospinning parts but, the collector or grounded glass plate is moved and collects the drawn-out fibers in a prescribed path determined by X and Y coordinates (writing).

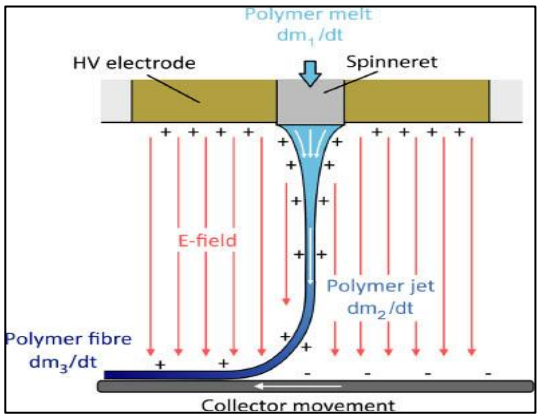

Figure 8: MESW method. The molten polymer is fed through a spinneret due to pressure. The HV electrode (including spinneret and melt) becomes charged and the collector becomes a ground. This draws out the polymer in a Taylor Cone formation. The collector plate directs where the polymer effectively "writes." [28]

This therefore creates very fine porous fibers useful as scaffolding material or as a mesh to enhance mechanical properties in biomaterials. The limitations include the building material which must be able to be melted, limited height due to the fibers becoming insulators, and poor consistency due to not matching the collector speed and fiber draw rate.

2.6 Bioinks

As important or even more important than the process are the materials being printed. Traditional 3D printing relies on melting polymers, but fragile biocompatible materials and cells are not likely to survive the standard heating and extrusion process. While methods such as extrusion have been modified to treat cells and other materials in a gentler manner, a specific encapsulation is required to be able to print such materials and maintain the desired shape after printing. The materials or mixture of materials which contains programed structures and geometries containing biomaterials and/or living cells is named a bioink. [29] Currently most bioinks are based on hydrogel biopolymers, such as collagen, gelatin, hyaluronan, silk, alginate, and nanocellulose. [29] These biopolymers can mimic the cellular environment while also providing an environment in which cells can multiply.

2.6.1 Bioink Categories

Five basic categories of bioinks can be described: structural, sacrificial, functional, supportive, and 4-dimensional. [29] Structural bioinks can form the shape/infrastructure of the print but are not sufficient for a functional tissue print. Sacrificial form channels which can later be removed and replaced with vascular networks, cell migration paths, or nutrient diffusion. Functional bioinks direct biological functions and promote cell differentiation. These include bioinks with mineral compositions for bone analogous materials such as OsteoInk. Supportive bioinks provide mechanical support for the printed materials or cells. 4-dimensional inks respond to

external stimuli. When stimuli are applied the shape, function, or structure of these bioinks changes. [29] The composition of the biologically compatible material inside these bioinks varies, as do the type of cells. Bioinks can be combined in various compositions until the desired structure, function, and shape can be achieved.

2.6.2 OsteoInk

The main bioink used in this project is OsteoInk from regenHU. OsteoInk is a calcium phosphate-based paste which is close to the natural composition of human bone minerals. It is intended to be used in 3D bioprinters for tissue engineering applications of bone, cartilage, or as a scaffolding material (Figure 9). This bioink can be combined with other bioinks to achieve different properties and functions. The mechanical properties depend on both the shape of the printed structure as well as the curing process used. It is intended as an experimental bioink as most of the properties and functions achievable by this bioink are not published. [30]

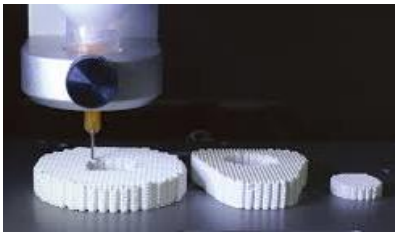

Figure 9: OsteoInk Matrix formations are created from OsteoInk paste. [30]

3 Methods and Their Implementation

3.1 Virtual Models of the Human Ear Ossicles

Obtaining the virtual models of the ossicles is the obvious first step in the project. Open-source models of the human ear ossicles were found on the University of Antwerp's website [17] ready for download. These models were created and released in many different file formats for educational and research purposes as described in one of their publication. [5] The models were obtained with an initial resolution of 2048 x 2048 pixels with 34.4 μm pixel and voxel size. [17] Once compounded, the researchers at the University of Antwerp created the STL files which represent a decently detailed 3D image of the ossicles according to the creators. [17] To verify this claim, other published literature was consulted as comparison. Studies such as the comparison made by Zhang et al. show that the details of the ossicles were well preserved in these models. [31] Other studies, such as the correlation of malleus and incus measurement by Wendell and Creighton, were also used to confirm that the proper shape and details of the ossicles were present in the virtual models used. [32]

Files of the models were available in formats such as .SURF that represent coordinates but, these files would only be useful in bioprinting if built or programmed directly into G-code. STL files were already created and subsequently utilized in this project as these were already prepared in a 3D format. 3D files were also required as the models could not be printed exactly as given and had to be modified for scale before being printed. This would not have been a problem if the scale matched the requirements of the printers and allowed coordinate system files to be used directly.

As previously discussed, STL files are just the exterior shell of the structure. Several modifications were thus required to the STL file. The primary modification required was for scale: the downloaded files were found to be oversized by a magnitude of 100x as compared to the anatomic object, making them impossible to print. Several programs were investigated for this task. MeshLab and Meshmixer were two programs which were able to successfully scale the model to the appropriate size without having to change the format of the STL file. BioCAM, which is the official software of the regenHU printer, was also able to successfully scale the model. The models created through different software were compared and analyzed.

3.2 Size of Ossicles

Size of the ossicles was determined by consulting available publications. Measurement points were taken from the work of Wendell Todd and Francis Creighton in their comparison of human ossicles. [32] The upper end of the spectrum was used for printing as the bigger sizes are more noticeable if details were printed correctly. The size of the nozzle in the extrusion printhead used is .20 mm, which means that layer thickness is able to print layers as thin as .20mm and should theoretically not have any problems with the small prints.

3.3 Model Editing and Modification

In order to provide further model altering besides the size, such as partial scaling of certain sections, more in depth editing of the STL models has had to be performed. As it is difficult to edit the STL file directly, the file requires transformation to an editable solid figure CAD file. To complete this operation, the surface has to be joined and then converted into a solid body. The first step in this process was converting the STL object to different surfaces, joining these surfaces together, and then using the join features of a CAD program to create a single file. This

process was completed in both CATIA and FreeCAD software. Models were compared from both software suites for differences.

3.4 Slicing and Infill

As an STL file is just the surface definition, the interior fill is software created during the slicing process. Infill amounts are easily adjusted through software settings. The manufacture of OsteoInk suggests that infill amounts are dependent on the shape printed and require adjustments for each model printed. If the infill is not sufficient, the material will collapse either during printing or while curing. No instructions or previous research has been completed using OsteoInk or a similar material in creating similar sized 3D structures in curved shapes. As the manufacturer suggested, settings of rectilinear and an 80% infill were used as the starting point, and variations of the infill patterns and fill rate were attempted. Observations on the outer shape and printing process were made.

3.5 Printing Path and Visualization

Before physically printing, the software created printer nozzle path was observed. This path is created without regards for material properties or other conditions which could affect the printed materials. The print head selected and nozzle diameter dictates which settings are available for use and how many layers are created. For this reason, the models were visualized both as a whole print and also as individual layers before printing.

3.6 Printing Process

The experimental printing process took place in several stages. As the regenHU printer (Figure 10) allows for fine control of most aspects of printing such as the layer height, pressure applied and feed rate, the first step involved figuring out the proper settings. We determined that the proper combination of software and hardware settings heavily influence the final outcome of the print, therefore getting the right mix of settings was crucial.

Figure 10: regenHU's 3DBioFactory bioprinter with biosafety hood. [30]

To prove the concept, the printer's ability to perform the desired operations, and to determine the initial settings, the first prints were conducted in NIVEA hand lotion ('crème') due to its consistency, reliability, availability, and nontoxic cleanup. This provided preliminary settings for the activity used with OsteoInk.

Different nozzles are also available to be used on the regenHU printer. Unlike OsteoInk, NIVEA crème does not cause any variations with different nozzles as it is of a more malleable consistency. No data was available on the use of OsteoInk with a straight gauge or conical nozzle.

The interplay between layer height settings in the software and the physical limits of the nozzle sizes had to be determined experimentally. If the pressure is set too high, then the layer thickness

will create a low-resolution print. If the layer thickness is too low, then the print will still be compromised in terms of resolution and size and may even collapse. The software and hardware controls were used to create the most robust print when properly tuned. If the layers are not sufficient, yet the thickness size is appropriate, then the software was manipulated into printing more layers by selecting lower printer nozzle size. The opposite was completed when required.

Most prints with NIVEA crème were done directly on a glass slides (unless using a scaffold, see below). Standard laboratory microscopy slides were used due to ease of sample removal, ease of cleanup, compatibility with almost any printable material, and availability. The glass slide was also used as it did not create any constraints to the printer which would have happened with a welled plate or a more complicated setup.

A heated printer bed is an option on the printer and if required can be used. Chilled lines are also available for use. Experiments proved that temperature did not affect OsteoInk or NIVEA crème and therefore these options were not used.

3.7 Post Printing Processing

Once all setting for printing with OsteoInk were figured out, the manufacturer's instructions were used for the post print processing of the ossicles. The manufacturer suggests an incubation period of at least four days for the material to cure. The printed ossicles were placed in an incubator for periods of four to five days. The prints were investigated for hardness, texture, and material mass determined in several instances. The exact material composition of OsteoInk is unique (and an industrial secret of regenHU), therefore no changes can be made to the chemical composition of OsteoInk to improve or modify its material traits. All optimization to this material must thus come from the incubation period and printing settings.

3.8 Support Materials

It was not known if a scaffold or an additional support material was required when printing OsteoInk. Several options were available for the creation of the support structure. After discussing this possibility with regenHU, the idea of using a hydrogel was brought up. PCL plastic through an extrusion method was also an option. The third option was the installation of the Melt Electrospinning Writing (MESW) unit and using that method to print a scaffold.

The PCL and Pluronic support materials were printed simultaneously with the OsteoInk, or printed individually. When printed individually, the OsteoInk was dispensed into the support structure after its 100% completion. The feasibility of the material was determined at the printing stage by observing if: (i) the support material kept its shape during both the simultaneous or individual material printing; (ii) after printing, by comparing the printed model to the virtual model; (iii) after curing, by both observing the shape of the support material and if the support was able to hold the OsteoInk.

3.9 Repeatability

The final objective of this project was to determine the repeatability and robustness of the process. To verify the size of the structure, ten prints were completed, and the mass of these prints compared. If the weight remained reasonably similar after curing, then the same amount of material has been dispensed and used in the structure. The support structure was not used in this measurement to only measure the amount of the actual OsteoInk dispensed. A visual check determined if the structures or textures were compromised in any way. If the G-code can be

extracted and used with the same nozzle sizes and pressure settings, then the same print will be able to be conducted on a different machine. G-code was analyzed and the regenHU specific commands were determined. Any machine should be able to complete a print if the machine is operating properly and able to maintain the pressure and speed required.

4 Findings and Analysis

4.1 Modeling and Pre OsteoInk Printing

A 3D model of a "virtual surgery" was initially created to show the usefulness of the printed ossicles. This is an example of ossicle trauma and represents a possible use of the models created. This modeling was created by taking the individual STL files, converting them into solid models, and then arranging the ossicles in the proper biological formulation. The models were converted to solid bodies in FreeCAD. The conversion allowed for further modification in Fusion 360 CAD software. Figure 11 shows the final outcome of this modeling.

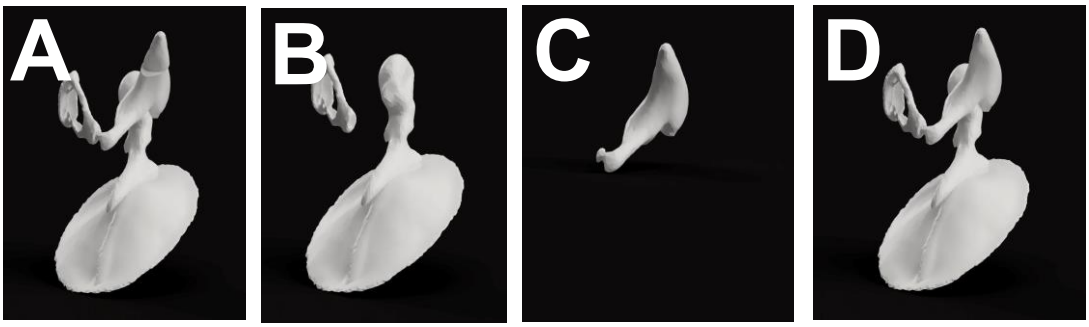

Figure 11: Virtual modeling of damage and repair in the middle ear. Simulated incus trauma (A), followed by its removal of the remaining portion (B), the design of a replacement (C) and the assembled reconstitution of the ossicles assembly. Such 'virtual surgery' may help the physicians in planning and executing difficult operations with very small objects like these ossicles.

The initial printing allowed us to determine the baseline settings for the extrusion printhead. This was the main printhead used throughout the project and the one used for the printing of the ossicles. As a surrogate bioink; NIVEA crème was used due to the reasons given in the Methods section. The consistency of NIVEA crème is of a paste, which means that unlike plastic material which quickly cures into the desired shape, the paste will settle and combine into one compounded print. NIVEA crème proved to be a sufficient proxy material which saved the waste of more expensive OsteoInk as well as time from tinkering with settings.

However, the printing with NIVEA crème was initially challenging as it required dialing in the pressure settings. In the regenHU instrument these are controlled both by an external pressure valve and also through software settings. The software settings were mostly used as a guide to create the virtualized print but were also found to have "black box" effects through unknown software calculations in BioCAM for the number of layers, print path, final speed, feed rate, and slicing. The external pressure determines the amount of material flow from the syringe tip, which when adjusted accordingly with the speed, determines the actual layer thickness. One of the characteristics of material "printability" is that it has to dispense without clogging. The initial settings for the external pressure sensor were determined by allowing the NIVEA crème to flow out of the nozzle. If the pressure was too low, the material would clot and stick to the upper end of the nozzle. This would result in multiple paths when printing and ultimately in failed prints. The minimum pressure was achieved when the NIVEA crème fell straight down. Figure 12A shows the representation of a single layer path. Figure 12B is the interposing of all layers of the

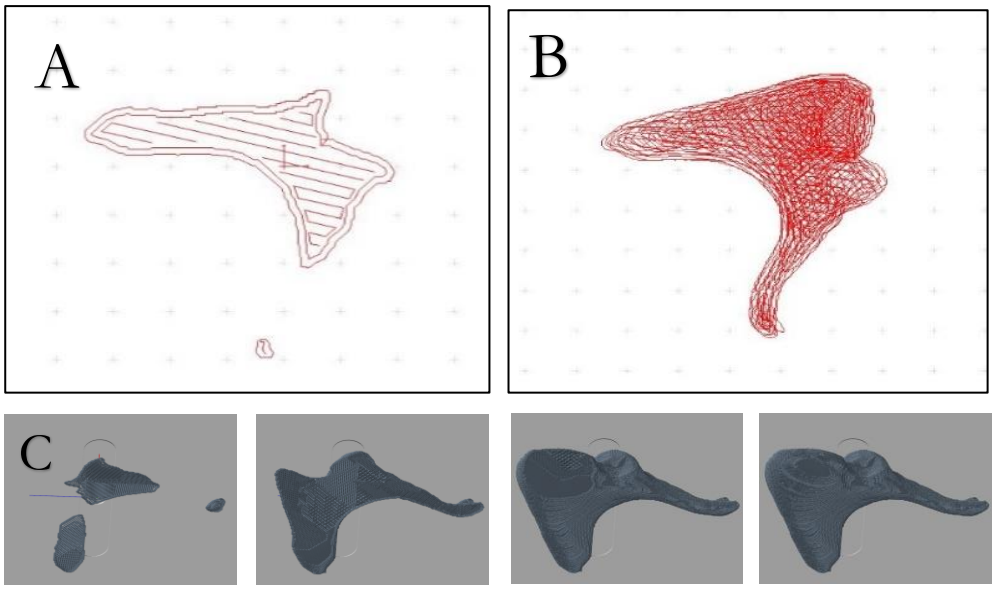

Figure 12: Virtual Layers. A: Single layer print from regenHU's BioCAD program. **B:** Total filling trajectory of a print as generated by the regenHU's BioCAD program. **C**: Layer progression. Each layer builds upon the previous layer.

print. Figure 12C shows how the layers build on each other. A higher number of layers result in a higher resolution as more details are able to be resolved per layer but could also create lower resolution and failed prints due compounded errors if the proper settings are not applied.

Figure 13 shows the first success print of an ossicle model with NIVEA crème. It was oversized and of the stapes, but it provided the baseline for further printing. As noticed in Figure 13, the layers have not been applied in an even manner. This is due to inconsistencies between the external and software pressure. The software pressure determines the number of layers and the calculated print path. The actual print is therefore determined by the interplay of these two settings. The best combination was found to be 4.0 Bar for software pressure and .156 mmHg for external pressure.

The rectilinear infill at 80% was deemed to be sufficient for NIVEA crème. Other infill patterns available include: honeycomb, two component grid, concentric, and Archimedean chords with infills ranging from 1% to 100%. Component grid, concentric, and line infills were attempted with ranges from 20% to 80%, but no actual difference in the print with NIVEA crème was found. 80% with the rectilinear infill was chosen as the best setting, as it provided the most infill at a sufficiently fast print speed. The highest amount of infill was chosen as this would theoretically provide the strongest structure due to incorporating the higher amount of material. In depth research on the infill amount and infill pattern was not completed as it was not deemed necessary. Cost of NIVEA crème is also negligible for such small objects as the ossicles. The consistency of NIVEA crème and of OsteoInk is of a paste, meaning that unlike plastic material which quickly cures

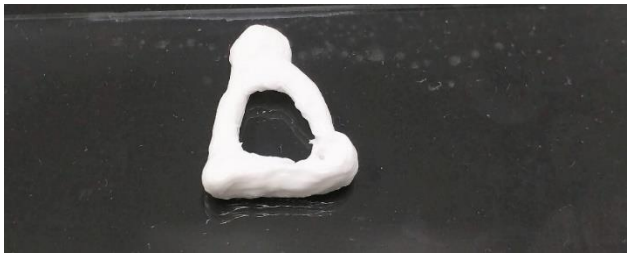

Figure 13: First successful print with the regenHU printer. The object is oversized compared to a human stapes and many details seen in the virtual modeling of the stapes are not evident in this print.

into the desired shape, the paste will settle and combine into one uniform print. This further diminishes the need for a specific infill.

Feed rate, i.e. the speed of the machine, was also an important factor when printing. The final feed rate chosen for NIVEA crème was 15 mm/s. Faster rates were attempted, but the pressure could not keep up to create consistent prints. Air pressure was limited to .285 mmHg by the built-in external pressure regulator. As the size of the ossicles is quite small and the quantity of objects printed is quite low, there was no need to have a high speed production. The quality of the print is much more important than the speed which dictates generally slower printing. The consistency of the NIVEA crème also does not allow for a higher speed than 60 mm/s with an 80% infill on the regenHU printer due to the X axis and Y axis movement causing the printer to transmit too much vibration and negatively alter the print. As there was no need to print at such high speeds and the printing was done at much lower speeds, this has not been an issue. No further investigation was required or performed.

4.2 Settings Transfer to OsteoInk

The settings obtained through experiments with NIVEA crème formed a solid base for working with OsteoInk. Figure 14 shows the final list of the settings used for OsteoInk. The external pressure setting had to be increased for OsteoInk due to the grittier consistency of the paste. Infill density was lowered due to the higher pressure causing more material to flow out of the nozzle. As previously discussed, infill density was not a factor in successful print, and 30% for OsteoInk was found to work well. Nozzle choice had to be different with OsteoInk. All other settings proved to remain very close to the settings determined for NIVEA crème. Settings and process for printing with OsteoInk is described in the next sections.

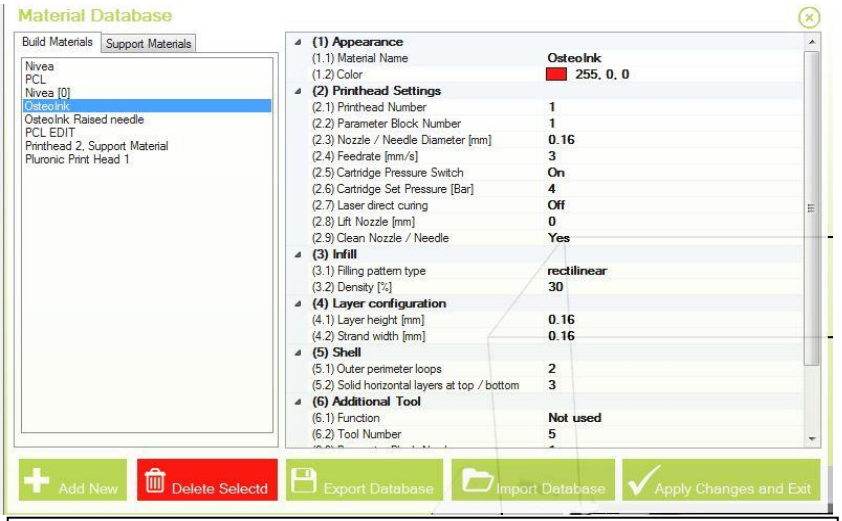

Figure 14: Final Settings for printing OsteoInk. Nozzle Diameter, feed rate, pressure, filling pattern, density, layer height, strand width, outer perimeter loops, and solid horizontal layers at the top/bottom were updated. Lift nozzle was attempted. Screenshot taken from regenHU's BioCam software. The baseline settings were taken from NIVEA crème and then optimized through experimentation.

Unlike the previous NIVEA crème prints, the size of the ossicles was taken into considering when printing with OsteoInk. Research revealed that variations in size of individual human ossicles are possible (Figure 15). Values on the upper end of the scale were chosen. To determine feasibility of printing, anatomically relevant, not exact, scale was required. Figure 15 shows the information used to size the printed ossicles. The size used in the repeatability study of the incus

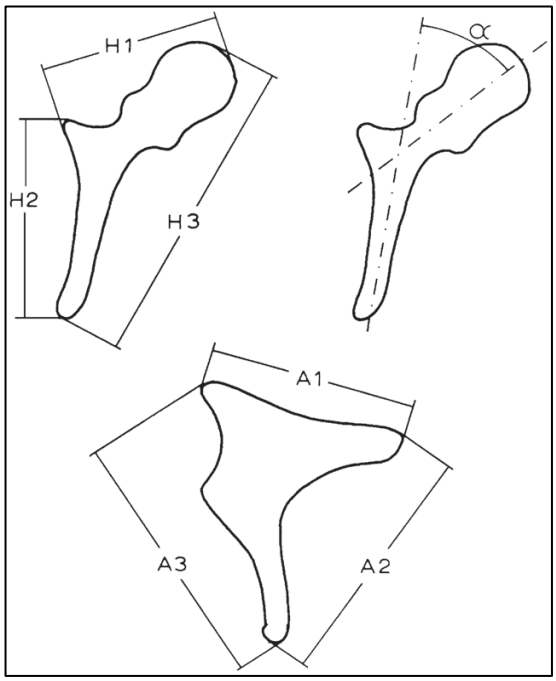

TABLE 1. REPEATABILITY AND DISTRIBUTION OF EACH MEASUREMENT

Parameter	Statistical Repeatability (r)	Practical Repeatability	Mean ± SD	Minimum	First Quartile	Median	Third Quartile	Maximum
Mastoid area, right (cm^2)	0.91 (N = 41; 95% CI, 0.84 to 0.96)	76% ≤2.0	9.0 ± 3.6	2.4	5.7	9.6	12.4	14.2
Mastoid area, left (cm^2)	0.91 (N = 41; 95% CI, 0.84 to 0.96)	83% ≤2.0	9.6 ± 3.6	2.0	7.5	10.0	11.6	18.0
H1,* malleus, right (mm)	0.84 (N = 33; 95% CI, 0.69 to 0.92)	63% ≤0.2	4.9 ± 0.3	4.4	4.7	4.8	5.2	5.4
H1, malleus, left (mm)	0.79 (N = 27; 95% CI, 0.58 to 0.90)	67% ≤0.2	4.9 ± 0.3	4.1	4.7	4.9	5.1	5.9
H2, malleus, right (mm)	0.96 (N = 33; 95% CI, 0.92 to 0.98)	93% ≤0.2	4.8 ± 0.4	3.9	4.5	4.8	5.0	5.5
H2, malleus, left (mm)	0.92 (N = 27; 95% CI, 0.83 to 0.96)	81% ≤0.2	4.9 ± 0.5	4.2	4.5	5.0	5.1	5.4
H3, malleus, right (mm)	0.88 (N = 33; 95% CI, 0.77 to 0.94)	72% ≤0.2	8.0 ± 0.4	7.1	7.7	8.0	8.2	8.8
H3, malleus, left (mm)	0.83 (N = 27; 95% CI, 0.65 to 0.92)	67% ≤0.2	7.9 ± 0.4	7.2	7.7	8.0	8.2	8.8
H malleus angle, right (°)	0.78 (N = 33; 95% CI, 0.59 to 0.89)	87% ≤5	135 ± 7	124	132	139	142	154°
H malleus angle, left (°)	0.94 (N = 27; 95% CI, 0.87 to 0.97)	100% ≤5	135 ± 8	120	129	136	140	153°
A1, incus, right (mm)	0.77 (N = 35; 95% CI, 0.58 to 0.88)	80% ≤0.2	5.0 ± 0.3	4.5	4.8	5.1	5.2	5.7
A1, incus, left (mm)	0.72 (N = 33; 95% CI, 0.50 to 0.85)	60% ≤0.2	5.0 ± 0.3	4.4	4.9	5.0	5.3	5.5
A2, incus, right (mm)	0.88 (N = 35; 95% CI, 0.77 to 0.94)	74% ≤0.2	5.9 ± 0.4	4.8	5.6	6.0	6.2	6.7
A2, incus, left (mm)	0.74 (N = 33; 95% CI, 0.53 to 0.86)	75% ≤0.2	5.8 ± 0.3	4.7	5.6	5.8	6.0	6.4
A3, incus, right (mm)	0.80 (N = 35; 95% CI, 0.63 to 0.89)	65% ≤0.2	6.5 ± 0.3	5.6	6.3	6.5	6.7	7.0
A3, incus, left (mm)	0.82 (N = 33; 95% CI, 0.66 to 0.91)	60% ≤0.2	6.5 ± 0.3	5.1	6.2	6.5	6.7	8.1
Malleus mass, right (mg)†	Not done (N = 28)	Not done	26.5 ± 1.9	22.5	25.2	26.1	28.1	30.7
Malleus mass, left (mg)	Not done (N = 26)	Not done	26.3 ± 2.4	21.2	25.0	25.8	27.9	30.4
Incus mass, right (mg)	Not done (N = 33)	Not done	29.1 ± 3.1	24.4	26.8	28.8	30.9	37.4
Incus mass, left (mg)	Not done (N = 33)	Not done	29.4 ± 2.8	25.1	27.5	29.0	31.4	36.3

Repeatability is depicted both by Pearson product correlation coefficient r and by practically relevant value. Each mean, standard deviation, and 5-number summary is average of 2 independent measurements, each made by single observer.

*See Fig 3 for measurement scheme.

†In sequence of measurements of ossicles, weighing was done last; some specimens had been lost. For 28 right mallei weighed, mastoid area 5-number summary is 3.9, 7.2, 10.4, 12.8, and 14.2 cm^2. For 26 left mallei weighed, mastoid area 5-number summary is 2.8, 8.0, 9.9, 11.6, and 18.0 cm^2.

Figure 15: Range of possible ossicles dimensions. Chart shows study and variations between individual sizes. [31]

printing was 10.27 mm total width, 11.97 mm total length, and 4.47 mm total height. The virtual models were successfully scaled in MeshLab, Meshmixer, and BioCAM software while maintaining the STL format.

4.2.1 Nozzle Choice

The regenHU printer utilizes a standard syringe cartridge for material loading with a Luer lock system. With this setup, numerous nozzle options are possible in a wide variety of sizes. Unlike OsteoInk, NIVEA crème is a highly viscous substance and any nozzle provided adequate results. As OsteoInk is close to the composition of bone it contains minerals and is a coarse material which creates issues with dispersion nozzles. The nozzles tested were: 22 gauge (0.41 mm inner diameter), a 23 gauge (0.33mm inner diameter), and 25 gauge (0.25mm inner diameter), as well as a plastic 27 gauge conical nozzle with a 0.2mm inner diameter. Starting with the smallest gauge, the 25 gauge nozzle, OsteoInk would not flow out of the nozzle, even at the highest possible pressure of 0.285 mmHg. The highest diameter nozzle, the 22 gauge nozzle, theoretically the worst choice as it will provide the lowest resolution, also did not flow OsteoInk. Sizes between 22 and 25 gauges were not tested as these would also not have allowed OsteoInk to flow. The conical (plastic) nozzle was the only one which allowed the OsteoInk to flow. This most likely has to do with the straight through portion of the non-conical nozzles creating a blockage due to the friction caused by the coarse OsteoInk. The conical shape provides an initially wider and a more gradual descent for

Table 1: Comparison of Tested Nozzle Sizes

Nozzle Type	Gauge Size	Inner Diameter (mm)	OsteoInk Flow
Straight	22	0.41	No
Straight	23	0.33	No
Straight	25	0.25	No
Conical	27	0.20	Yes

improved flow. For this purpose, the conical nozzle is the best choice as it is also the smallest diameter available and creates the smallest layers with the highest detail in the print.

4.2.2 Pressure

After the determination of nozzle size, the exact pressure which could be used had to be determined. NIVEA crème provided the baseline and starting point of .120 to .130 mmHg. As OsteoInk has different material properties than NIVEA crème, more pressure had to be applied. The precise amount was found to depend on environmental factors and the amount of time the OsteoInk was left outside of an air tight container. The lowest amount of pressure for day of use OsteoInk, as OsteoInk used in the same day it was taken out of the original packaging, was found to be 0.138 mmHg. After one day, it was found that pressure had to be set to .141 mmHg. After two or more days, the pressure had to be set to 0.157 mmHg or higher. Humidity was also a factor in determining the pressure required as OsteoInk absorbs water causing it to prematurely cure. Higher humidity creates the requirement for more pressure.

As room temperature, humidity, and amount of time exposed to the air influence the flow characteristics of OsteoInk, the exact pressure requirements must be determined immediately before printing. The range used to print ossicles has been approximately 0.130 to 0.160 mmHg. The best way to determine if the pressure is sufficient was the process discovered through experiments with NIVEA crème. Selecting the extrusion print head and turning on the pressure and dispense features allows for the flow of material from the nozzle. A steady stream going directly downwards in the Z axis direction will provide the print seen in the visualization. If the pressure is set too low, the material will not dispense or form a loop and attach itself to the nozzle. If too much pressure is selected, then the lines created by the extrusion printhead could be compromised by becoming oversized. Theoretically, the size of the nozzle restricts the material and therefore the print layers and strands will not be of the same size. This makes it seem as selecting too much pressure is not as big of a concern as having too little pressure. In practice, it was discovered that too much pressure creates an oversized layer. The use of a plastic nozzle could result in a deformed tip after extended periods of high pressure, especially with a coarse material as OsteoInk. It is therefore the best choice to set the pressure close to the lower threshold in the range.

4.2.3 Feed Rate

The feed rate is simplistically representing the printing speed and determines how quickly the print head moves around it's predetermined path. It is measured in millimeters moved per second. In combination with the pressure, feed rate determines how much material is deposited. The small size of the ossicles and small number of prints dictated that the slowest possible feed rate was the best idea. As discovered from printing with NIVEA crème, a feed rate which was set too high would cause too many vibrations and damage the print, therefore a low federate of 3 mm/s was used as a safeguard. Higher rates could be achieved if desired, but there was no need during this project.

4.2.4 Filling Pattern

Due to the consistency of OsteoInk it was determined that filling pattern does not influence the exterior shape of the printed construct. The automatically created filling patterns of rectilinear was used as it was proven to be sufficient. Figure 16 shows the interior of an incus print with the rectilinear filling pattern. Honeycomb, two component grid, concentric, and Archimedean chords were also tested with OsteoInk and no observable changes to the texture or external structure were observed. The process created was found to work with all filling patterns. If the material properties are required to be altered for future research, then the infill pattern should be investigated. Otherwise settings used were found to be sufficient.

Figure 16: Infill prints. The left shows the rectilinear interior fill pattern while the right shows the completed print of an incus. Printed in OsteoInk.

4.3 Initial Print

Even if the settings of the OsteoInk were able to be dialed in, the first completed prints of the incus did not yield the appropriate results: the material did not support itself but deformed and collapsed outwards. This was due to not having a base to provide support before curing for the paste like OsteoInk. For the material to support itself, the first layers until 0.560 mm were removed from the model using the BioCAD program. This was accomplished by viewing each individual layer and finding a flat base. Any layer below 0.560 mm was found to not be useable. Figure 17 shows the prints which were accomplished through this method. This provided a flat layer for the material to settle. This process was done on an experimental trial as there is no method which could be implemented to devise the best flat layer which could be used for printing. This does create the problem with not being able to print out the whole structure without the use of a support structure. This experimentation determined the need for a support structure.

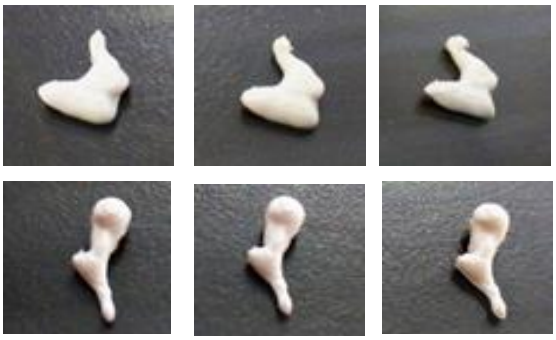

Figure 17: Three completed prints of the malleus and incus on a glass slides before curing. Support structure was not used as layers were omitted from the print to obtain a flat base. The desired structure and the proper external texture were achieved.

4.4 Curing

The structure of bone is hard stiff and relatively fragile, but that does not translate well to printable structures. A printable material must be malleable. Since this is a biocompatible material it also can't be melted and extruded like in traditional polymer based additive manufacturing methods. A solution to this limitation is to have a material which can be modified after being printed.

Figure 18: Printed malleus and incus after curing for five days. No discernable changed in structure or exterior texture observed.

OsteoInk is such a material and according to the manufacturer, requires a curing process to obtain the bone analogous physical properties. After printing, the printed shapes were cured in an incubator at 100% humidity for a minimum of four days. Heat and moisture caused the OsteoInk to transform from a paste into a hard material. Figure 18 shows the printed malleus and incus after five days of incubation. Spending more than four days in the incubator was found to not make a major difference as the material can only absorb a set amount of moisture and no more material changes could occur afterwards.

4.5 Reproducibility

Figure 19: Repeatability prints. The ten incus prints after curing. Size and texture are repeatable though minor differences in external texture can be noticed.

To see if the process is repeatable and robust, the process was repeated ten times. The ten incus prints were incubated for five days at 100% humidity. The mass of the prints was then compared and can be seen in Table 1. The printed ossicles can be seen in Figure 19. These prints were also completed with the flat base starting at the .560mm layer. This was done in order to not have to create a support structure as only the actual prints were desired to be measured and not the differences caused by a support structure or another material.

As shown in Table 2, the mass of the prints is between 0.044 and 0.050 grams. The variation between prints is low at a relative standard deviation of 5.7%. The weight differences show that the amount of material used in each print is comparable and that the process can be repeated. This also proves that the dimensions achieved for each print are reasonably similar. Further research is required to determine how much tolerance should be acceptable from each print based on human anatomy. In terms of the goal of this project which is to create a process for printing ossicles, the chosen difference of less than 10% is considered acceptable.

Table 2: Mass of 10x Incus Prints

Sample	Mass (grams)
1	0.044
2	0.051
3	0.044
4	0.045
5	0.044
6	0.046
7	0.047
8	0.044
9	0.048
10	0.050

Relative Standard Deviation (%)
5.67340196
Mean (grams)
0.0463
Standard Deviation
0.002626785

4.6 Experimental Setup

Printing of the ossicles was conducted with a regenHU 3DBioFactory bioprinter. The material used was OsteoInk also from regenHU. 3D models were edited human ossicles freely available online for research purposes. [17] No modifications were made to the material. The material was loaded in the printer in 3CC cartridges Luer lock plastic conical 27 gauge nozzle. FreeCAD, CATIA, and MeshMixer were used to edit the models. RegenHu's BioCAD and BioCAM programs were also used to edit the models. RegenHu's HMI (Human Machine Interface) program was the host program used to send the G-Code to the bioprinter and control the printer. Settings used can be found in Table 3.

Table 3: Final Settings for Repeatability Prints

Software Nozzle Diameter	0.16 mm
Actual nozzle Diameter (Physical Nozzle)	0.20 mm
Feed Rate	3 mm/second
Infill Pattern	Rectilinear
Infill Pattern Density	30 %
Software Layer Height	0.16 mm
Software Strand Width	0.16 mm
Actual Layer Height (Physical Nozzle)	0.20 mm
Actual Strand Width (Physical Nozzle)	0.20 mm
Outer Perimeter Loops (For Shell)	2
Solid Horizontal Layers at Top/Bottom (For Shell)	3

4.6 Support Structure

As discussed in previous sections, a support structure was required in order to print the round shaped structures. Support structures are common in additive manufacturing, therefore the BioCAM program used in this research incorporates an automated feature which creates a useable support structure based on the shape of the supported object. The support structure can be seen in grey in Figure 20. An issue encountered with this feature was that BioCAM does not allow any modification of the generated support model and is unable to export the support structure's STL file to an external program to be modified. Different materials were tested with this automated structure.

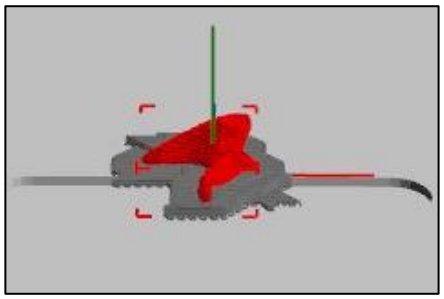

Figure 20: Design of an ossicle support structure. Red color represents the model while the grey area represents the support structure created in BioCAM.

4.6.1 PCL Support Structure Through Fused Deposition

As this process has never been previously attempted and OsteoInk has not been used in this manner before, the required material for a support structure was unknown. PCL was initially chosen as the support material because it is a common material in the additive manufacturing field and easy to print. The chosen method was heat extrusion at 100°C from pellets with a .2 mm inner diameter deposition nozzle from a direct pellet extrusion printhead.

In practice the software was not optimized to print both the support structure and another material (ossicles in this case) at the same time despite having and using two separate print heads.

During the print it was observed that the print head nozzles were crossing into the materials deposited in previous layers, which caused the two materials to become intertangled and the previous layers to be unusable. Layers were printed simultaneously based on Z-axis position rather than calculated based on best time to print. It was also attempted to raise the nozzles after printing each layer, but the nozzles still touched the other material during the print. Glass (from the glass slide used to print on) was also not able to support the PCL as the initial layers were not able to grip and remain on the slide. Laboratory grade adhesive tape was used as an adhesive surface for the PCL. The use of an adhesive backed substance introduces concerns as the adhesive may not be biocompatible and may even degrade to the point of mixing with OsteoInk.

The next attempt was to print the support structure first and then try to place the OsteoInk inside this structure. As the BioCAM program used to create the support does not permit exporting the STL file, the G-code itself had to be edited. Parts of the code which use the extrusion print head for OsteoInk were removed. Z-axis were not altered as the G-code started with the support structure and this allows for the structures to remain unmodified. This process did not create any problems while printing. The ossicle structure, in this case the incus, was placed in the center, the same spot as the support structure, and printed. The support structure was able to be printed successfully in PCL. However it was not possible to print the OsteoInk in the support. The printing nozzle with the OsteoInk collided with the already printed structure. The only way to avoid this issue would be to manually alter the G-code, which would be very time consuming, and thus unfeasible. The structure printed can be seen in Figure 21.

Figure 21: PCL scaffold attempted through extrusion printing. Individual PCL Support structure printed on adhesive backed material. Glass slide was not able to be used for this print.

The resolution of the extruded PCL support structure was also not sufficient for the small size of the ossicles. The outside texture was found to be altered due to the practical resolution of the PCL printing. Another issue encountered with PCL was the adhesion of the plastic to the OsteoInk. Once printed and cured, it was difficult to separate the OsteoInk from the plastic without breaking the print due to the brittle nature of cured OsteoInk. Because of these two significant reasons, printing PCL with the direct pellet deposition print head was not deemed a viable solution.

4.6.2 Pluronic Support Structure

Another commonly used support biomaterial is Pluronic (also known as 'Poloxamers', a class of materials with different molecular weights). As a hydrogel this material can be combined with water in different proportions to obtain different consistencies. The most commonly used concentration is 20-40% Pluronic in water. To achieve a structurally sound support for bioprinting, a higher concentration of Pluronic must be used. This creates problems because as the concentration increases Pluronic becomes less fluid and viscous. Pluronic also has the unique quality of becoming more fluid as temperature decreases. This creates the challenge of storing and handling the high concentration Pluronic while maintaining it at or near 0°C. A chilling system for the cartridge inside the bioprinter is available, but in practice this system was only able to maintain the temperature at 7-9°C which was not helpful during printing.

Another challenge was creating the actual hydrogel solution. Pluronic is sold as a powder, and different mixtures create different concentrations of the substance. In order to create high

concentrations of 500mL batches, the Pluronic has to be kept cold and then constantly stirred for 8-24 hours depending on the concentration, stirring speed, and temperature.

We initially attempted to prepare a 60% Pluronic solution in a cold room which maintained the temperatures of 7-9° C, but this was too thick to be able to be created using a standard laboratory stir plate and magnetic stir stick. We discovered a successful method involving the freezing of the Pluronic solution -20°C freezer. Once frozen, the Pluronic is placed in the cold room and back on the stir plate. As the solution thaws out it becomes less viscous and the stir stick is able to start stirring. If the solution becomes too thick to be stirred, it can be placed back in the freezer and frozen again. When the solution is clear and does not have any undissolved particles, it forms a transparent gel.

The microvalve (inkjet) was used to print the support structure as the extrusion printhead on the regenHU machine was reserved for the OsteoInk. The 60% concentration was first attempted but was found to clog up the valve. 50% and later 40% concentrations were also attempted, but the issue persisted. The highest concentration which was able to be used without clogging the valve was found to be 35%. This was the concentration used for the remaining prints.

The microvalve was adjusted until a steady stream was able to be deposited. This required a pulse (valve opening time) with a duration of 800 ms. The dosing distance was set at .200 mm. No other settings have a major influence on the microvalve. Pressure is not very important as the deposition of material is controlled by the valve rather than pushed out through a nozzle. The chilled cartridge holder for the Pluronic cooled the mixture down to a measured 7-9°C. This was found to be unnecessary as the prints did not take much time to be completed and the temperature change was negligible during the printing. The most effective method was to place the Pluronic on ice until used. Ice also kept the Pluronic at a lower temperature than using the chilled cartridge system.

In a similar method to the PCL support printing, the structure was created in BioCAM and the automatically created process was used to print the support structure and ossicle simultaneously. The microvalve printhead is shorter than the pellet deposition printhead and also operates from a higher Z-axis distance. Despite these differences, the same issue with the interference of the nozzles as seen with the PCL and pellet extrusion printhead occurred.

The G-code was also edited to print only one structure at a time. This resulted in a structure which printed without issue. Printing the OsteoInk though caused the structure to collapse as the OsteoInk extrusion nozzle penetrated the Pluronic structure. Combined with the mass of the OsteoInk, the support structure created by Pluronic did not hold the OsteoInk in the proper shape. Therefore, Pluronic and similar hydrogels were deemed not be to useful support structures. Figure 22 shows the unsuccessful print using with Pluronic as a support structure.

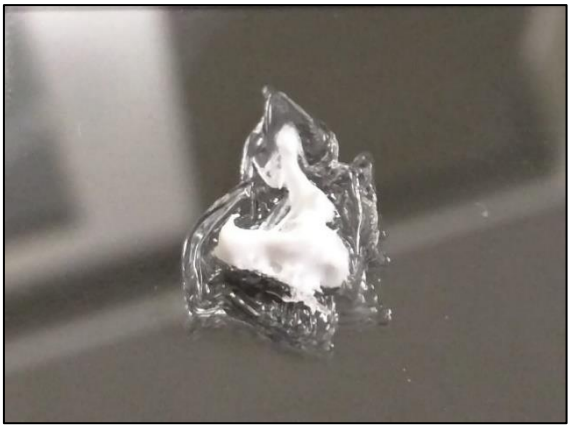

Figure 22: Pluronic scaffold obtained by inkjet printing. Unsuccessful printing the support structure using Pluronic and then printing the OsteoInk. The Plutonic was not able to support the OsteoInk.

4.6.3 PCL Support Structure Through Melt Electrospinning Writing (MESW)

To address the issue with resolution and printhead nozzles destroying previous printed layers, the MESW unit of the regenHU instrument was used to print the required support structure. The flat layer which was previously found to be an acceptable base for the ossicles, .506 mm for the incus or .607 mm for the malleus, was used as the attempted height of the designed support structure. As MESW operation has not been implemented in the BioCAM, the support structure had to be created by altering the G-code to be compatible with the MESW unit. The MESW was found to require extensive testing for placing the PCL strands in the expected pattern on the collector plate.

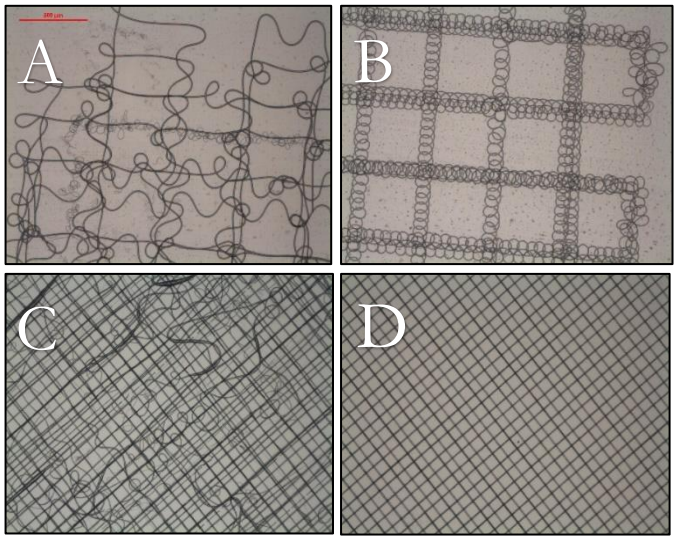

Figure 23: MESW visualized with stereomicroscopy. (Red bar for scale: 500 μm) A: Initial print with fan. **B:** No fan and low feed rate. **C**: High feed rate and high collector plate distance. **D:** High feed rate and low collector plate distance. Red line (500 μm) for reference.

MESW depends on pressure, voltage, distance to the collector, and feed rate. Figure 23 displays the micrometer scale fibers through images taken by stereo microscopy. Figure 23A shows the initial print with MESW. The strands were collected in a random matter. This was later determined to be from the use of the fan inside the fume hood and safety cabinet. Figure 23B is the resulting print without any interference from the fan. As it can be seen, the fibers were collected in a circular pattern. We determined that this was caused by a slow feed rate and a high collector plate distance. The printer head was lowered, and feed rate was quadrupled. The results can be seen in Figure 23C. This resulted in straight collected fibers, but the fibers couldn't be maintained throughout the print. The PCL was clogging in the nozzle which caused the spinning of the fibers to be disrupted. Once the pressure was slightly increased to .225 mm HG, the fibers were able to be maintained for 10-15 layers. The result of this is shown in Figure 23D. The fibers were placed in perfect position, but it was not possible to stack past 15 layers. Figure 24 shows the completed support structure printed with MESW. A useable volume for a support structure was not achieved due to the incredibly fine layers and limited layers of the MESW process. A physical size optimized structure was explored but determined to be not necessary as it was discovered that the MESW unit must operate at high feed rates.

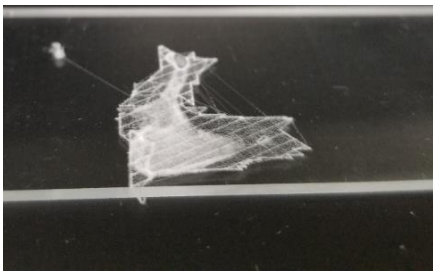

Figure 24: MESW scaffold. The proper shape was achieved, but the required volume was not able to be achieved.

5 Discussion and Conclusions

5.1 Discussion of Method

The objectives of creating a process for printing customized ossicle models have been met. The combination of using a new method to create these models with a novel material has resulted in a process which can be repeated for other bones of similar size. This process has proven to allow alterations to the virtual models, the OsteoInk material was able to be utilized for these prints, and the process has proven reproducible. The machine code and settings determined in this project can be transferred to other bioprinters. This allows the process to be shareable and eventually to reach the end goal of 3D bioprinting of patient implantable ossicles.

The method chosen starting with the modification of computer models, printing a partial model, and then adding improvements for the printing has proven effective. The models chosen were validated to be anatomically correct through a literature study. These models provide a reasonable starting point for shape and size alterations in further research. The surrogate testing material, NIVEA crème, was found to be a cost and time savings method of starting the printing. The material used, OsteoInk has proven to cure without causing any defects in the printed shape after the process has been completed.

The creation of the support structure was more difficult than anticipated. While the software available, BioCAM, could create an automated support, it was not able to provide sufficient support, and could not optimized for simultaneous printing alongside non polymer based materials. Custom made hydrogel material, Pluronic, was attempted to be used as a support material but did not provide enough mechanical support. This was a challenge as the creation of the support structure was initially believed to have been possible through PCL based extrusion but became too complicated to be solved for this thesis. Less time should have been spent on materials for the support structure and more time should have been focused on MESW based support structure as that process showed the most promise.

5.2 Discussion of Findings

5.2.1 CAD

Published open source models were used for this project. Kuru et. al was just one of the sources that have proven the models used to be anatomically correct. [7] Different populations have proven to have large variations in sizes of ossicles. [33-35] The size chosen for this project was proven to be a reasonable representation on the upper range of the size.

The ossicle models were able to be modified for size and scale as STL files in MeshLab, Meshmixer, and BioCAM software. The models were also successfully converted to solid files which can be further modified in commercial CAD software such as CATIA, SolidWorks, or FreeCAD for the scaling of only specific parts of the model or more complex alterations. The models created with different software suites and from the two modifications methods were compared and no discernable differences were observed.

"Virtual surgery" was also completed. Virtual ossicle models were created in order to visualize real-life situations which would otherwise require ossicle repair. The printed ossicle is inserted into the chain rather than the more typical bone cement repair of the damaged ossicle. This process would create a standardized repair rather than the current on the spot surgical repair. [6]

The G-code was able to be extracted and printer specific commands were able to be identified. This left the X, Y, and Z coordinates, feed rate, and pressure settings; commands which can be applied to any other bioprinter. Commands exclusive to bioprinters from other manufactures can be added to repeat the process of printing the ossicles. It would have been beneficial to learn and alter G-code earlier in the project as a lot of time was spent configuring software and searching for settings in the proprietary BioCAM and BioCAD software. G-code would have also helped during troubleshooting activities.

5.2.2 3D Printing Method

Given the nature of the materials used in this project, extrusion was the best choice for printing the malleus and incus. NIVEA crème proved a great surrogate material for establishing the baseline settings of extrusion contact printing. Before starting this project, it was not known if extrusion based bioprinting was even able to be used to print the small sizes required of the malleus and incus, but it was determined to be a feasible method. Printer settings such as feed rate, external pressure, software pressure, infill percentage, nozzle, and infill pattern were determined. These settings have proven to be reliable and repeatable. The curing process resulted in the expected hard yet relatively brittle ossicles. As the process of printing whole ossicles has not been previously attempted, the process created in this project will be very useful for further research as it can be directly applied and used to print most other bones of similar sizes. Previous research in the use of 3D printing with calcium phosphate has been mainly for bone tissue repair, scaffolding for cells, and scaffolding for drug delivery. [36]

The stapes was not able to be printed using the process devised for the malleus and incus. This is due to the small size of the stapes and requires more investigation into the process or material. Extrusion methods did not provide enough resolution with the 0.2 mm nozzle in order to print a stapes with a reasonable size and resolution.

5.2.3 Material

Besides the use of a surrogate bioink for testing setting, OsteoInk was the main bioink used in this project. It was tested and proven feasible as a material for bone like prothesis created by 3D bioprinting. The manufacturer of OsteoInk recommends it mainly as support material and as a matrix for cell growth in hard tissue applications, no mention of using OsteoInk as a prothesis is made. [30] Previous research into the use of 3D bioprinting and calcium phosphate has been focused on matrix formulations used to grow cells, drug delivery devices, or bone patch devices. [36, 37] Therefore any development of OsteoInk as bone prothesis has been previously unexplored. As a biomaterial close to the composition of human bone, OsteoInk possess the desired biological properties for implantable ossicle prothesis.

Issues that remain to be solved include the soft nature of the printable OsteoInk and the issue of physical size. A size on the higher end of the human malleus and incus was chosen as it was the easiest to print, but it was discovered that smaller sized ossicles, such as the stapes, are significantly more difficult to print. Larger sized bone prothesis will ultimately just require larger scaffolds, but smaller sizes will not be able to be printed using the process proposed in this project. New techniques will have to be developed to print the stapes. In order to mitigate the issue of the coarse OsteoInk damaging the nozzle, a water based binding system could be attempted for the inkjet microvalve printer. [38]

The application of OsteoInk has been successful as a guideline but requires more research to be able to be used as a definitive solution for ossicle implants. OsteoInk requires a scaffold to be

printed in the desired shape, but a more useful solution will be printing without the need for any support. The goal of this project is the quick and easy printing of ossicle replacement prosthesis which can be created without the need for specialized 3D printing skills. The main problem lies in the creation of a paste which can be extruded. The higher the viscosity, the more likely that a print can be created without the need of a support structure, but that necessitates lower resolution due to a larger dispensing nozzle. This problem might be solved if extrusion was not used. Encapsulation in a hydrogel and printing with a light-based technique such as stereolithography could be explored. [39, 40] We did not have access to a stereolithography printer to attempt this process.

We also noticed that OsteoInk is brittle. Different mixtures of OsteoInk with other bioinks could be created to counteract this property. The washing and curing process is the biggest factor in determining the final properties of pure OsteoInk and further investigation is required in optimizing the curing process. The OsteoInk material was also found to be too brittle for the small size of the stapes. Small parts of the incus and malleus were easily broken; therefore the stapes would not be able to survive much handling after curing. Further investigation is required in determining mechanical properties for ossicle implants, especially of the stapes.

5.2.4 Scaffold

Creating a support structure for the OsteoInk is not as trivial as believed at the start of this project. Traditional 3D printing allows for polymer scaffold which can later be physically removed from the main print with mechanical force. Automated generation of support material is common and effective in traditional 3D printing, but this is not possible with 3D bioprinting yet. This rules out traditional scaffold methods and most software which creates automated support structures. While specialized Bioprinting software, BioCAM, was available and utilized to create a support structure, the software was optimized for polymer based printing and scaffold structures. The software was able to use different printing heads, one for the building and one for the support material, but the interaction between the two printing heads was not a consideration when using soft materials such as pastes. The printing heads and nozzles crossed into the previous deposited layers causing both structures to be compromised. BioCAM software was not found to be sufficient for the requirements of this project.

Materials used for the support structure were not made specifically for printing OsteoInk. As OsteoInk was developed mainly as a matrix material, the manufacture believed it would effectively support other materials. It was discovered that OsteoInk is a dense material which requires a material with optimal mechanical properties to be used as a support. Polymers are the obvious choice due to the high strength to weight ratio, but a practical method for combining a biocompatible polymer with OsteoInk was not found. The main issue was the intersection of the print head nozzles of the support material with that used for OsteoInk. Specialized software must be created to predict nozzle movement and guide the printheads without intersecting previously deposited layers. Hydrogels had the same nozzle interaction issue and were also unable to provide proper support to the OsteoInk.

MESW is a promising start to a support structure for OsteoInk as it combines the mechanical strength of a polymer (PCL) with very high resolution. However, more research is required to overcome the issues preventing the buildup of large volumes of materials (the electric field is disturbed by the polymer fiber buildup) using this MESW technology. Possibilities include further optimization of the process which could result in up to 50 layers. [41] MESW could also be combined with hydrogels in a hybrid system which would give hydrogels the mechanical strength and resolution of MESW while retaining their biological compatibility and volume.

Hydrogels would be reinforced with MESW fibers to form a composite biomaterial similar to cartilage. [42]

5.2.5 Quality Assurance and Control

Printing ten models using the exact same settings and on the same day to minimize environmental factors has proven that the models have around a 6% difference in mass when compared with each other as seen in Table 1. Research gives values of malleus between 21.2 to 30.7 mg. [32] This dictates that the printed parts need to have a lower infill percentage and possibly lower curing times. The general shape was found similar, but not exactly the same. Small differences can be observed in Figure 19. This was mostly due to the last printed layer maintaining adhesion with the OsteoInk remaining in the cartridge. When the printer head moves up a strand is pulled from the cartridge and drops on the print. This was not observed in every print and therefore the shape and the mass are minimally affected. This could be resolved by altering the G-code to lower or completely stop the air pressure to the cartridge towards the end point of the final layer. More research is required to obtain the perfect print.

The process created for printing the ossicles has been defined and can be exported to any compatible bioprinter. The G-code was able to be extracted and all coordinates can easily be transferred to other printers. If all settings expressed in this project are followed, ossicles printed from OsteoInk can be easily created.

5.2.5 Problems and Disadvantages to Method

Several disadvantages have been discovered when printing with the proposed method. The main disadvantage is the total time required to complete the process. Printing may not take a long time, but curing the ossicles into the bone like material required a minimum of four days in an incubator. Another disadvantage is the equipment requirements. Different material combinations and/or ultraviolet cured material could dramatically lower the process time, but more research is required for such materials. Reliable 3D Bioprinters are currently relegated to research institutions and major corporations due to the high costs. Incubators are also costly to own and maintain. New models and new manufactures are constantly lowering the cost of bioprinters through new innovations in the field. Costs will surely go down in the near future as exemplified by polymer based 3D printers. The reliance on OsteoInk is also a drawback as regenHU could discontinue the sale of the product without notice. More investigation into the use of similar material is required, but at this time no similar bioinks have been identified.

5.2.6 Project Progression

As this project was novel and experimental, the proposed outline created at the start of the project could not be followed exactly. However, the research of methods and information gathering stages of the program was achieved without any unanticipated issues. The computer modeling and editing of the models was also accomplished without any issues. Printing the model involved more trial and required a few more days than planned. The major issues occurred when attempting to print the scaffold. As previously discussed, it turned out to be more difficult and time consuming than expected. A simple wax or polymer scaffold used in traditional 3D printing was not possible due to the specifics of the instrument, so novel solutions were attempted with biocompatible materials. More research into the materials of both the main print and the scaffold are required. Improved method could also help solve the issue. More research also needs to be completed in order to print the stapes. Due to the small size of the stapes, contact extrusion was

not possible to be used. More time during the progression of the project should have been spent on attempting to solve these two issues.

5.2.7 Research Questions

Question 1: Are the available ossicle models appropriately sized and comparable to natural human ossicles in shape and features and could these models be modified?

The 3D computer models were comparable to models and medical images published in research articles. The models were able to be converted to solid models from STL files and edited in CAD programs.

Question 2: Are the available bioprinting methods adequate for printing ossicles?

Yes, contact extrusion was an adequate method for printing the malleus and incus in the proper size. The stapes requires a different method and/or process.

Question 3: Will a scaffold or supporting structure be required during the printing of the ossicles?

Yes, a support structure was required for printing ossicles. OsteoInk cannot support itself before being cured in the required ossicle shapes.

Question 4: Which materials and methods are appropriate and practical for such a structure?

PCL was the best material while MESW was found to be the most promising method for printing the support structure. This provides the required mechanical support while maintaining the required exterior detail of the ossicle bones. More research is required into implementing this method.

Question 5: Can the process and the product of the printed ossicles meet the expected quality level, and could this process be repeated at the same quality level?

The quality was found to be acceptable and exterior detail of the computer model was preserved after the printing and curing process. Ten prints were created and these matched within a reasonable margin to each other to prove repeatability.

Question 6: Can this process be transferred to other bioprinters?

Yes, this process was found to be transferable to other comparable bioprinters on the market. The settings discovered combined with the G-Code can be easily repeated on any comparable bioprinter. Models are freely available on the internet. [17]

5.3 Conclusions

All Research Questions were addressed, and progress has been obtained in most of them, generating a workflow for printing hard tissue such as bones on a small physical scale. Overall, we prepared and modified the ossicle computer models, produced simulations which showed the possible use of the ossicles, created visualizations of the printing process, and tested if the models could be printed in OsteoInk. This process was proven to be repeatable on the regenHU bioprinter. The G-code was extractable and when combined with the settings given in this project, there should be no problems repeating this work on the same scale on other bioprinters.

While the application of OsteoInk has been successful as a starting point for bioprinting implants in a highly biocompatible material, more research is required in creating a complete process. Multiple objectives have been determined over the course of this project to require more attention.

The stapes has proven to be too small to be printed using the process tested here. A new system or a new print head is required to successfully print a stapes. A light (UV or laser) based printer or a perhaps a microvalve capable of handling OsteoInk could be used. Extrusion was not found to be the most effective on a small scale, especially when combined with a coarse material such as OsteoInk.

More CAD work is also required in the future, specifically for support structures. Creating custom support structures which could be used with the MESW printhead will be highly beneficial in the prototyping phase. Automation in CAD development would also be highly beneficial to the end goal of the project. Creating software which could take medical images and compound these into 3D models would be very useful for creating customized prosthesis and avoiding CAD editing. CAD software could also be used to create blood vessels or nutrient channels inside the ossicles.

OsteoInk currently requires a scaffold to be printed in the desired shape. A more useful solution to 3D printing ossicle will be printing without the need for any support structure. The eventual goal of this project is the quick and easy printing of ossicle prostheses which can be created without the need for specialized 3D printing skills. The higher the viscosity, the more the likely that a print can be created without the need of a support structure, but that necessitates lower resolution due to a larger dispensing nozzle. OsteoInk can be combined with other biomaterials and with mechanically stiffening compounds to remove the need for a scaffold. This is major undertaking and requires the creation of new material combinations as well as mechanical testing of completed and cured prints. Materials such as alginate, collagen, or nanocellulose can be considered and mixed with OsteoInk to achieve this. Research has already been started in scaffold free bone tissue engineering. Some of the novel processes such as the soaking process for the repair of bone through the creation of composite materials could be integrated into 3D bioprinting and/or incorporated into the OsteoInk printing process. [43]

Further investigation in the repeatability of prints is also required. This requires research of the printing and curing process to find root causes for the current discrepancies. Further research should aim for reaching a goal of below 1% mass and size differences between ossicles. As OsteoInk is also influenced by humidity and temperature, environmental factors should also be studied to better understand repeatability.

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