DEVELOPMENT OF A BIOCOMPATIBLE AND DISSOLVABLE TYMPANOSTOMY TUBE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009

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To my wife Amy, and my parents, Amy and Robert

ACKNOWLEDGMENTS

The culmination of 30 years of my life has been quite a journey. This journey could not have been completed without the love, support, and guidance I have received from my parents. Their commitment to my education and well-being through tough times has been unwavering and it is this assurance that has propelled me to achieve my goals.

I would also like to thank my committee members Dr. Christopher Batich, Dr. Malisa Sarntinoranont, and most importantly Dr. Patrick Antonelli who have all helped to steer and motivate me to complete my PhD. I would also like to acknowledge Dr. William Ogle for filling in for Dr. Batich on my committee for my dissertation defense. Their combined creative guidance and technical support throughout this process has been second to none. Moreover, the completion of my doctoral dissertation would not have been possible without the help and guidance I have received from my advisor Dr. Roger Tran-Son-Tay. For more then 7 years he has been a true supporter of my research and has helped me achieve my goals of completing my doctorate. I would like to acknowledge all of their time, effort, and work propelling me throughout my graduate studies.

Furthermore, I would like to acknowledge the support I have received from my fellow graduate students. Without them this process would have been quite arduous. Jessica Cobb, Chessy Fernandez, and Jennifer Jackson have been immensely helpful in working out difficult problems and helping me to re-think design parameters. I would also like to thank Cecile Perrault for her more than 12 years of support throughout my entire undergraduate and graduate career.

Finally, I reserve my most heartfelt appreciation and love for my wife, Amy. The magnitude of her devotion and her love of both my strengths and weaknesses has been one of the most important driving forces in my life. Her desire for my happiness is evident by her never-

ending support and patience. The joy it has brought me has continued to be my beacon of hope during this process.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

DEVELOPMENT OF A BIOCOMPATIBLE AND DISSOLVABLE TYMPANOSTOMY TUBE

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August 2009

Chair: Roger Tran-Son-Tay
Major: Biomedical Engineering

Tympanostomy tubes (TTs) are designed to be implanted within the ear drum, facilitating pressure relief. TTs are commonly placed to treat recurrent acute and chronic otitis media with effusion in an estimated 2 million children yearly. The limitations of existing TTs include tube occlusion, premature extrusion, permanent hearing loss, and surgical removal. In addition, predictable and reliable ventilation is insufficient among existing tube designs due to variations in composition and geometry. As a result, the goal of this study was to develop a biocompatible tympanostomy tube (TT) capable of maintaining reliable ventilation as well as dissolving on demand.

The criteria for developing an alternative TT is sufficient mechanical strength, occlusion resistance, dissolution on demand, and biocompatibility. To this end, TTs were created using calcium alginate hydrogels. The mechanical strength of the gels was enhanced by altering the gel tube composition and manufacturing constraints. Furthermore, occlusion resistance was improved via application of albumin coatings. One of the unique features of these alginate gel tubes is their ability to dissolve after implantation with the use of sodium, magnesium, or potassium solutions, minimizing the need for surgical removal. Furthermore, biocompatibility

was maintained using clinically proven non-toxic ingredients and was corroborated using an in-vivo Zebrafish model.

Results show that biocompatible TTs can be made using an Alginate:PEG solution crosslinked with CaCl. Alginate and PEG (polyethylene glycol) have been used extensively in medicine with few side effects. Additionally, it was discovered that the ensuing tubes were significantly stronger than commercial silicone tubes (tension and compression) extending implantation durations. Furthermore, alginate tubes (coated in albumin) are less prone to occlusion than un-coated commercial stainless steel tubes while having the unique ability to dissolve on demand within one week after exposure to $NaHCO_3$ solution. Moreover, Zebrafish hair cell ototoxicity experiments indicate minimal toxicity levels after a 30 minute exposure to dissolved and filtered gel tubes. Therefore, dissolvable alginate gel tubes have the potential to limit complications associated with tube implantation.

In conclusion, TTs can be developed using calcium alginate gels. The mechanical properties of these gels can be manufactured to exceed those of commercial silicone tubes while providing a minimally invasive removal technique. In addition, biocompatible dissolution solutions ($NaHCO_3$) can be applied to dissolve the gel tubes on demand, reducing patient trauma. As a result, novel biocompatible alginate TTs can be developed that are capable of providing and maintaining reliable ventilation while dissolving on demand.

CHAPTER 1
RESEARCH PLAN

Introduction

Tympanostomy tubes (TTs) are commonly placed to treat recurrent acute otitis media and chronic otitis media with effusion [1]. These tubes are designed to provide ventilation within the middle ear. TT performance can be compromised by premature extrusion, occlusion, or prolonged implantation in up to 25% of cases [2-4]. These complications may result in patient morbidity and increased expense, possibly requiring surgery. As a result, the development of a reliable ventilation tube is critical for reducing complications associated with tube implantation.

Rationale

Prevention of TT occlusion, extrusion, and prolonged implantation has long been the focus of commercial research [5-7]. However, existing commercial tube designs have yet to successfully eliminate these complications. Currently, decreasing premature extrusion promotes prolonged implantation while minimizing occlusion increases the development of secondary infection [5,6]. On the other hand, a dissolvable and biocompatible TT could potentially limit the development of TT complications. Similar TTs have been created to reduce implantation duration and ventilation times [8-11]. However, unpredictable clinical conditions combined with a lack of physician control limit their widespread use.

Optimal TTs should retain middle ear ventilation until clinical symptoms have resolved, followed by simple removal. As a result, the focus of this research was on the development of a biocompatible, dissolvable, tympanostomy tube capable of providing reliable ventilation while limiting tube occlusions.

Objective

The overall objective of this project was to develop a biocompatible tympanostomy tube capable of maintaining reliable ventilation while dissolving on demand.

Specific Aims

1) Develop a calcium alginate substrate that can be made into a tympanostomy tube:

    a) Identify the parameters involved in developing a viable tympanostomy tube.

    b) Develop a procedure for producing both reuter-bobbin and "T" shaped alginate gel tubes.

    c) Maximize the mechanical strength of the alginate gel tube and compare it to a commercial Goode T-tube.

    d) Quantify mechanical strength after agar encapsulation.

    e) Quantify mechanical strength from exposure to ototopical solutions.

    f) Minimize plugging of the alginate gel tube and compare it to a commercial stainless steel reuter-bobbin tube.

2) Optimize the gel dissolution components for complete dissolution:

    a) Optimize the dissolution components (additive solution and dissolution solution) for complete dissolution.

    b) Quantify the effects of dehydration on the dissolution rate of the gel tube.

    c) Quantify the effects of human serum albumin coating on tube dissolution.

3) Quantify ototoxicity of alginate gel tube dissolution using a Zebrafish model**:**

    a) Quantify ototoxicity from the dissolution products.

Significance

These studies have the potential to promote the use of minimally invasive biocompatible techniques while controlling tube removal and limiting the expense and prevalence of surgery associated with TT insertion.

CHAPTER 2
BACKGROUND AND SIGNIFICANCE

Tympanostomy Tubes as a Cause for Sequelae

Tympanostomy tubes (TT) are devices that are inserted into the tympanic membrane in order to relieve pressure caused by the buildup of otitis media during an ear infection. They can be composed of either metal (titanium, stainless steel) or plastic (fluroplastic, silicone) and can be "T" or "Bobbin" shaped [5]. Sixty to seventy five percent of TTs perform flawlessly. However, some will develop a secondary complication. Three common and potentially detrimental complications are tube occlusion (~25 %) [4], premature extrusion (~4%) [3], and prolonged tube ventilation (>2yr (2.7%)) [2]. These complications can lead to temporary or permanent hearing loss and result in the superfluous implantation of tubes. In most cases tube occlusion can be relieved medically with the addition of otic drops. However, some cases involve the replacement of the tube. In addition, the majority of tympanic devices extrude naturally as a result of the healing process but, premature extrusion prior to the resolution of symptoms can lead to a resurgence of infection and additional tube implantation. Furthermore, tubes implanted for extended periods of time require surgical removal, resulting in additional trauma.

Two important factors involved in the development of these secondary complications are geometric variability and composition. Currently there are >30 different styles of TT each claiming to have advantages over the others. However, very little clinical data is available to support these claims. For instance, larger diameter tubes have been shown to decrease the development of an occlusion, but they increase the likelihood of chronic tympanic membrane perforation and secondary infection [5]. In addition, tubes designed to remain implanted for prolonged periods decrease the prevalence of extrusion, but are subject to a higher incidence of

chronic perforation and surgical removal [6]. Furthermore, tube composition plays a significant role in cellular adhesion which can contribute to tube occlusion. Smoother surfaces such as those impregnated with silver oxide, or albumin (Figure 2-1) have shown remarkable results in preventing biofilm formation which leads to a decrease in occlusion frequency [5,7].

Further studies conducted by Lentsch et al. [6] measured the correlation between elective surgical tube removal and chronic perforation. His results show that 11% of the population who voluntarily remove their tubes develop chronic tympanic membrane perforations. Therefore, the surgical procedure of removing the tympanostomy tube may contribute to the development of a perforation of the eardrum. At the very least it subjects the patient to an additional surgical procedure. The ideal tube would simply spontaneously extrude from the membrane or become dissolved after resolution of symptoms (otitis media) [12]. In addition, there is evidence that shows that the presence of granulation or cellular adhesions on the tube increases the healing capacity of the membrane and decreases perforation development [6]. This indicates that a biocompatible environment surrounding the perforation will be useful in repairing postoperative complications associated with tympanostomy tube placement.

Alginate Gel as a Non-toxic Degradable Gel Scaffold

Calcium alginate gel has been used as a scaffolding material for skin grafts, as well as a successful drug delivery device for over 10 years. It is also biocompatible and has a very low inflammatory response. In addition, calcium alginate has the unique ability to cure under relatively inert conditions making it ideal for use in cellular encapsulation and growth [13-16].

Alginate is one of the main components in most of the large brown seaweeds. It is composed of alginic acid which consists of 2 monomers, β-D-mannuronic acid and α-L-guluronic acid. The individual monomers, as well as combinations of these two monomers, form blocks which outline the basis for the resulting polymer gel. The G-Block is composed of L-

guluronic acid, and the M-Block is composed of D-mannuronic acid, with the MG-Block composed of both L-guluronic acid and D-mannuronic acid. In terms of appearance the M-Block is relatively straight and has the consistency of a ribbon, whereas the G-Blocks stereochemistry aids in the formation of gels (Figure 2-2). It is the G-Blocks that are crucial when creating gels using calcium ions [13,17]. This is because the G-Blocks contain reactive carboxylic acid sites whereas the M-Blocks are non-reactive. In order to create a gel, divalent solutions including (Ca^{2+}, Sr^{2+}, Ba^{2+}) are added to the gel. Magnesium (Mg^{2+}), along with monovalent solutions such as sodium (Na^{1+}) or potassium (K^{1+}) do not form gels. However, in the presence of calcium, the carboxylic acid site of the G-Block is ionically substituted with calcium (divalent), and a link between two alginate strands can be made, forming a solid "egg-box" structure (Figure 2-2) [13].

The above procedure of adding calcium to alginate specifically in the form of calcium chloride is one of the simplest ways to create a solid gel. The calcium content within the gel rapidly increases within the first hour of gelation and levels off over time. Gel stiffness is increased by diffusion of calcium ions from the liquid solution to the forming gel via the carboxylic groups of guluronic residues. However, the resulting calcium chloride solution takes several hours to diffuse smoothly into large quantities of alginate solution. On the other hand, the use of low solubility solutions such as calcium citrate or calcium sulfate will form very smooth gels quickly as a result of their slow steady release of calcium. Furthermore, this addition of calcium ions to a solution of alginic acid will cause the viscosity to increase without the addition of more alginate or by increasing the molecular weight [17].

A unique property of alginate gels is their ability to rapidly dissolve from a solid back into solution. It has been shown that the chemical bond is reversible in calcium alginate gels, making

them ideally suited for rapid degradation in the presence of chelating or monovalent ions. The chelating or monovalent ions can include sodium (Na^{1+}) or potassium (K^{1+}) [18]. Moreover, alginate gels can be degraded with the use of acidic hydrolysis or with the use of enzymes, along with gamma radiation and ultraviolet light [19].

Furthermore, calcium alginate has a proven biocompatible track record, which is a function of the concentration and purity of the M and G blocks. In addition, natural alginate gels have low cytotoxic and hemolysis effects. Moreover, calcium alginate has been used as a patch for repairing tympanic perforations in the ear [20]. Prior to the use of calcium alginate, paper patches or a surgical tympanoplasty procedure were considered standard practice for repairing tympanic perforations. However, recent developments have focused on the advancement of implantable/injectable devices that act as a scaffolding over the perforation to promote healing. Weber et al. [20] has successfully created a biocompatible calcium alginate plug with the use of an injection-molding 3-D manufacturing technique. Histological results showed little to no toxicity associated with gel implantation. This further validates the absence of cytotoxicity as a result of graft placement and validates the use of alginate based implants for use as tympanic inserts [20].

Polyethylene Glycol (PEG) as a Dissolution Additive

PEG is similar to alginate in that it is a common polymer used for tissue engineered scaffolds and for drug delivery [21]. PEG was added to the alginate gel solution to alter the final gels properties and to facilitate dissolution. Alginate gel strength is a function of the available alginate in solution. The substitution of PEG for some of the alginate prior to crosslinking reduces the amount of alginate and can potentially limit the mechanical strength of the resulting gel. As a result, the PEG encapsulated gels have the potential to dissolve faster then pure alginate gels.

Ototopical Solutions Used to Treat TT Sequelae

There is a vast array of treatments available to treat tympanic complications ranging from topical antibiotics to invasive surgical procedures. A large majority of these treatments consist of the use of topical solutions. These include Hydrogen Peroxide (H_2O_2) [22-24], Sodium Bicarbonate ($NaHCO_3$) [22,23,25], Sodium Chloride [22], Vinegar [22,24], Ciprodex, and Ofloxacin[26]. Several of these topical solutions are unique in their ability to potentially degrade or assist in the degradation of calcium alginate gels. These include sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), and vinegar (CH_3COOH, acetic acid) which are used with little clinical evidence to clear tube occlusions [23]. The first two compounds contain sodium (Na) which, as stated previously, is one of the monovalent ions capable of dissolving calcium alginate gel back into solution. Furthermore, sodium chloride has also been shown to be non-ototoxic [22]. The third compound, vinegar, does not contain any of the aforementioned monovalent ions. However, it may behave as a catalyst accelerating the dissolution reaction with the production of carbon dioxide gas, when in the presence of sodium bicarbonate. The advantages of the above solutions are that they are widely available and have been used, with few side effects, in the treatment of ear sequelae [22-25].

There is however one known drawback associated with the use of these solutions. Sodium bicarbonate 5% in solution has been associated with minimal pain. In a clinical study using sodium bicarbonate to relieve tympanostomy tube occlusions, minimal pain was associated with administration. However, the pain was mild in all patients and did not hinder the treatment regime [23].

Use of Zebrafish as a Screen for Ototoxicity

Hearing loss due to ototoxicity is commonly determined by measuring hair cell function. Developments in alternative animal models have lead to the discovery of Zebrafish as a

promising candidate for measuring hair cell ototoxicity associated with medical treatments. There are many advantages for using Zebrafish as a screen for ototoxicity. The most important is that Zebrafish hair cells are structurally, and functionally similar to the inner ear hair cells of mammals. However, unlike mammalian hair cells, these cells are located externally along the lateral-line of the fish. Zebrafish also have typical vertebrate inner ears with both hearing and vestibular organs along with the external hair cells [27]. One advantage of using Zebrafish is that these external hair cells can be observed within the organism as a whole rather then cultured separately as in mammalian studies. Furthermore, Zebrafish embryos are optically clear, which promotes hair cell observation via microscopy and facilitates fluorescent labeling. As a result, the Zebrafish model allows testing of ototoxic samples, not just at the cellular level, but metabolic level as well. Also, neuromast hair cells have been shown to undergo apoptosis after injury using aminoglycosides in a similar manner to the way mammalian organ of Corti hair cells undergo cell death. Moreover, mammalian studies frequently generate false negatives since rodents can still respond to ambient sound after losing most of their high frequency hearing [28,29]. Additionally, Zebrafish are inexpensive to obtain and can be rapidly analyzed making them ideal for large sample sizes.

Hair cells convert mechanical sound vibrations into electrical signals that are carried by the auditory nerve to the brain. The external hair cells in fish (Zebrafish) are organized into clusters of 5-20 cells that are contained within neuromasts found along the lateral line and head. Each neuromast is composed of a ring of support cells that surround the sensory hair cells within the center. These neuromasts can detect frequencies of between 50-200 Hz and are primarily used to facilitate schooling, and to avoid predators. Also, lateral line hair cells exhibit selective uptake of fluorescent dyes including FM1-43 (via nompC TRP channel), DASPEI (2-(4-

(dimethylamino)styryl)-N-ethylpyridinium iodide) a fluorescent styryl dye, and Yo-PRO1, another fluorescent dye, making them ideal for rapid ototoxicity analysis [28-31].

The use of fluorescence to assess ototoxicity provides a quantifiable measurement of the effect ototoxic chemicals have on individual hair cells. The fluorescent intensity of the hair cells provides a measurement of hair cell viability. Furthermore, each fluorescent dye can selectively label a different part of the hair cell allowing for a more specific cellular analysis. For example: FM1-43 can selectively label only the cytoplasm of the hair cell while Yo-PRO1 will only label the nuclei (nucleic acid, DNA) of the cell, and DASPEI is a voltage sensitive dye which can label nerve fibers and neurons as well as hair cells of the lateral line neuromast and nasal epithelium [27,28,30]. More specifically, FM1-43, in Xenopus hair cell, labels organelles such as mitochondria, and rough endoplasmic reticulum within the cytoplasm and is inhibited by cation channel blockers such as neomycin and amiloride. On the other hand, Yo-PRO1 is a cyanine monomer dye capable of entering cells through large non-selective channels such as P2X7 receptors [32]. DASPEI (cationic) stains work by staining mitochondria within living cells. Under specific conditions the stain can accumulate within the mitochondria due to the development of negative potentials across the cell membrane. However, the DASPEI stain is not present (stored) in adjacent supporting cells, only in the nucleus of hair cells. This is because there may be differences in the ion channels and exposed carriers of both hair cells and support cells. Also, hair cell transmembrane potential is higher than that of the surrounding support cells and it is this membrane potential that has been shown to affect the uptake of other cationic dyes [33].

Under fluorescence the hair cells glow depending on their viability and can be graded based on florescent intensity. Typical results show a distinct decrease in intensity as toxicity increases [30] and under increased magnification, individual hair cells within the neuromast can be

counted and compared. However, it is important to note that drug susceptibility is dependent on cell maturation with 4 day and 5 day old fish showing marked differences. Younger (immature) neuromast cells are less susceptible to ototoxic chemicals and therefore can give false readings when compared to older samples. Studies have shown that individual hair cell maturation rather than larval maturation is the determining factor when observing stage-dependent differences. Therefore, it is important to maintain strict control over the treatment dosage and hair cell maturation when comparing across samples [32].

Prior to the use of Zebrafish, in-vitro cell lines cultured from mammalian and avian isolated hair cells were used. These in-vitro methods, however, are not capable of assessing the metabolism of the drugs by the organism or the potential causes of toxicity in that animal. As a result, many cell based assays fail expensive animal studies. More importantly, the function of hair cells depends on not just the cells themselves but the surrounding support cells, which are not present in in-vitro cell culture. Furthermore, cells cultured in-vitro have a limited number of target sites when compared to whole organisms and can not represent the diversity available in whole animals (ex. fish). In-vitro cell culture also requires the use of "fresh" primary cell lines with short longevity times [34]. In addition, up to this point, cell based assays have been based more on availability and not on specific function. Moreover, isolated cells are less sensitive to individual chemicals than whole fish, reducing their use in obtaining absolute safety levels [35]. Therefore, in-vitro cell culture may be inadequate for reproducing in-vivo conditions.

There are, however, some drawbacks to the use of Zebrafish as a model for studying hair cell loss. The hair cells contained in the neuromasts of Zebrafish do not contain stria vascularis or spiral ganglia, which are known targets of ototoxic drugs, nor do they contain perilymph and endolymph. In addition, unlike mammals, Zebrafish hair cells can regenerate after exposure to

ototoxic chemicals after 12-24 hours [28]. Also, lateral line hair cell death may be accelerated in Zebrafish when compared to mammalian ototoxicity results [32]. Furthermore, the morphological and physiological characteristics of neuromast hair cells are more similar to vestibular rather than mammalian cochlear hair cells [30]. Despite these limitations, the optical clarity of embryonic Zebrafish, along with the selective uptake of fluorescent dyes and reduction in cost due to rapid analysis of dose response assays, paves the way for measurable in-vivo assessment of hair cell death [29,30].

Absorbable Tympanostomy Tubes

The majority of commercial TTs are composed from either stainless steel, titanium, silicone, or fluroplastic and do not have the ability to non-toxically degrade within the body. Therefore, the duration of implantation is limited by the geometry of the tube and healing rate of the patient. However, several new techniques have been developed for manufacturing tubes out of absorbable polymers. Two polymer choices are poly (D, L-lactide-co-glycolide) (PLGA) and poly-bis(ethylalanate)phosphazene (P-PHOS) [8-11]. Both are biodegradable polymers that have a defined resorption rate. The potential impact of these devices is to eliminate or at least reduce the need to surgically remove displaced tubes or to help control retained tubes [8].

However, the main drawback associated with these absorbable polymers is the inability to control the rate of dissolution in-vivo. Once implanted, absorbable tubes have a pre-set resorption time, which cannot be altered. As a result, after tube absorption any incidence of recurrent otitis media with effusion will require tube re-insertion, increasing trauma to the patient[3].

The development of these degradable TT has been spurred by the often unpredictable nature of middle ear ventilation using conventional tubes. This is due to the extremely high rate of spontaneous extrusion (up to 90%) among commercial tubes resulting from the natural healing

process. Currently the most common solution to combat premature extrusion and promote extended ventilation is the use of “long-term” tubes. These tubes are designed to resist extrusion while maintaining ventilation over an extended period of time (> 2 years). Usually, patients requiring “long-term” ventilation have chronic or recurrent otitis media with effusion and require repeated tube implantation. The development of the Goode T-tube in the 1970s provided a device that resisted extrusion and offered long term ventilation. The Goode tubes and other “T” shaped tubes achieve this with use of an extra wide flange on their medial side [36,37].

There are, however, complications associated with the implantation of long-term ventilation tubes which limits their widespread use. These complications include the need to surgically remove the tube in up to 90% of retained cases, and the increased incidence of tympanic membrane perforation post extrusion [37]. Furthermore, tympanosclerosis (membrane thickening) or granulations surrounding the tubes can form and cause bleeding. Also, cholesteatomas (abnormal skin growth) can develop as a result of epithelial entrapment due to localized atrophy of the tympanic membrane [9,37]. On the other hand, the development and implantation of a biocompatible and dissolvable TT will eliminate the need for surgery and can further reduce the complication rate.

The significance of our approach is that it focuses on attacking one of the most prevalent postoperative complications associated with tympanostomy tube implantation (reliable tube ventilation). The use of dissolvable calcium alginate gel implants may reduce patient trauma by reducing the need for additional surgical procedures. Also, with more effective implant devices, the frequency of hospital visits for post operative care will decrease, and therefore the amount of anesthesia administered to children will decrease. As a result, the recovery time will be substantially quicker than with previous techniques.

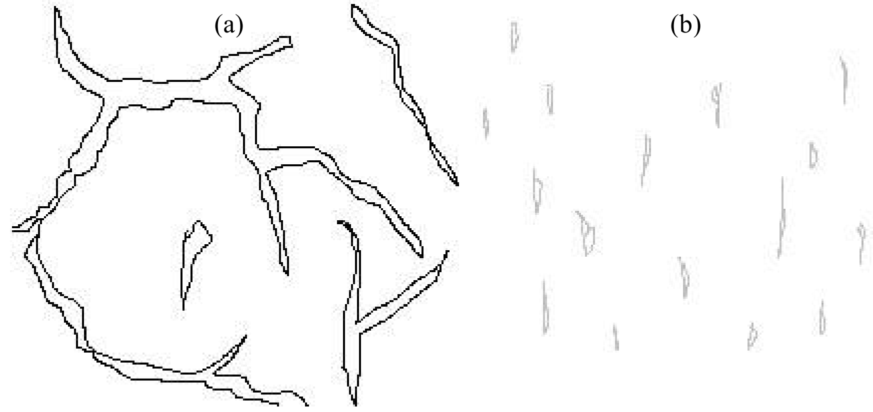


Figure 2-1: Surface structure of tympanostomy tubes (at ~ 500x magnification). (A) Uncoated, large deep cracks in the tube surface. (B) Human serum albumin coated, small surface cracks.

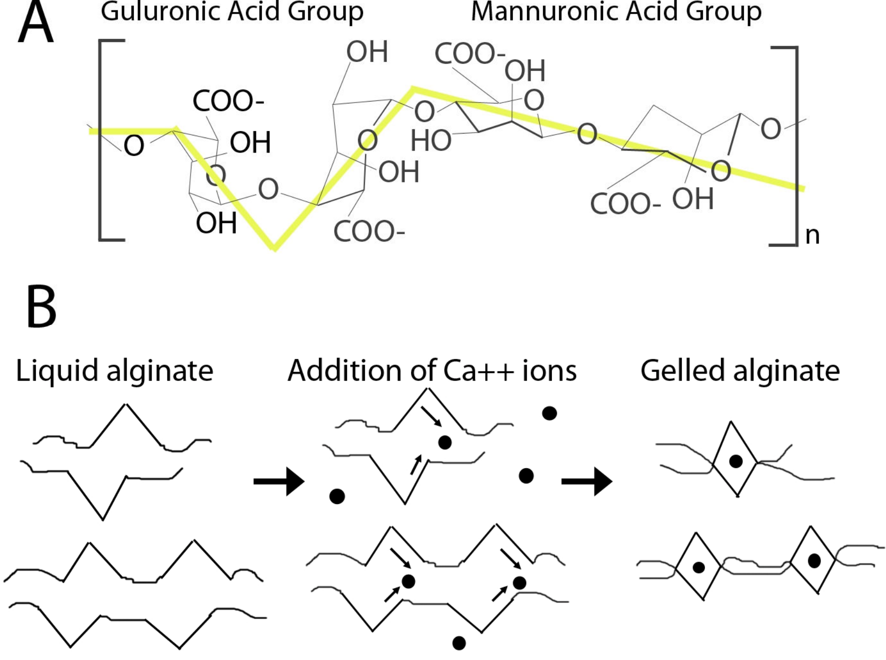


Figure 2-2: Calcium alginate molecular structure. (A) Guluronic and Mannuronic acid groups. (B) Egg-box structure for gelled calcium alginate.

CHAPTER 3
MATERIALS AND METHODS

Gel Preparation

For the following experiments, alginate (gel) was prepared by mixing 3 grams low viscosity alginate powder (Sigma, A-2158, Macrocystis pyrifera (Kelp), M/G ratio ~1.5) into 100ml deionized water (DI) using a magnetic mixer (Corning Stirrer/Hotplate, PC-420) at 50°C for 1.0 hour. The alginate gel has an M/G ratio of 1.5-1.67 and a molecular weight of 50 x 10^3 [38,39]. This mixture was allowed to rest over night resulting in a 3% concentration of alginate [39].

Calcium chloride (curing solution) was prepared by mixing 1.1 grams of calcium chloride (Sigma, C-2661, Calcium Chloride Anhydrous), with 100ml of DI water at 50°C using the magnet mixer. This mixture resulted in a 0.1M solution of CaCl. 0.5M, 1.0M, and 2.0M solutions were created by adding 0.266 grams, 0.599 grams, and 1.265 grams of calcium chloride to 6ml of 0.1M CaCl prepared solution[13,40].

Polyethylene Glycol (PEG) (dissolution additive) was prepared by mixing 0.335 grams of PEG (Sigma, P-3640, MW 3350) with 100ml of DI water at 50°C. This mixture resulted in a 0.001M PEG solution [41].

Sodium bicarbonate (Baking Soda, $NaHCO_3$) (dissolution solution) was prepared by mixing 0.1008g $NaHCO_3$ into 12ml of DI water at 37°C. This mixture resulted in a 0.1M $NaHCO_3$ solution. 0.5M, 1M, and 2M solutions were created using 0.504g, 1.008g, and 2.016g of $NaHCO_3$ in 12ml of solution [22,23]. The above concentration ratios were chosen due to their use in previous studies as well as their ability to rapidly crosslink each other into a solid gel [18]. Prior to mixing, the solutions were filtered using a 0.22um filter (Fisherbrand , 09-719A). The filter also strains out any particulate matter contained within the alginate gel, PEG, $NaHCO_3$, or

CaCl and creates a smooth homogeneous solution [39]. All remaining solution concentrations were described as necessary.

In-Vitro Ear Chamber

An in-vitro ear chamber was used to quantify tube plugging. The chamber was designed to simulate the flow of a fluid (mucus simulant) and air through a tympanostomy tube.

Ear Model Construction

A model ear chamber was machined from commercially available acrylic (Lexan). This included ports for the delivery of mucus and air into a larger chamber overlying a replaceable latex diaphragm that held the TTs (Figures 3-1, 3-2). Figure 3-1 (section view of the ear chamber) demonstrates the flow of mucus and air within the chamber and through the tube. Figure 3-2 describes each of the ear chamber components. Figure 3-2C-D-E-F-G are shown in section view in Figure 3-1. Figure 3-2B (foam seal (Darice Foamies Sheets, 1144-13, 2.2mm x 2.2mm x 2mm thick, Strongsville OH)) provides a seal for the front surface of the tympanic membrane apparatus (Figure 3-2C, 3-2E) and prevents air/mucus from leaking. Figure 3-2A (compression plate and machine screws) maintains chamber integrity while providing a port for visual correlation of TT status with chamber pressure. Figure 3-2H corresponds to a fenestration on the side of the air delivery port (Figure 3-2F) allowing for measurement of chamber pressure via a pressure transducer.

A replaceable diaphragm, "tympanic membrane," consisted of a thin latex membrane (Fisherbrand powder free latex exam glove, Fisher Scientific, Pittsburgh, PA) (Figure 3-2C, 3-2E) supported by a rigid metal washer (Steel, 15.875mm outer diameter, 12.7mm inner diameter, 0.254mm thick). The membrane was glued to the surface of the washer (Elmer's Multipurpose Spray Adhesive, E452, Columbus, OH) and then a tympanostomy tube was inserted through a fenestration in the center.

Mucus Delivery

Flow of mucus was assumed to result from both production of mucus by the middle ear mucosa and influx of air from the Eustachian tube during swallowing, sneezing, and nose blowing. The rate of mucus production was assumed to be linear. However; it is “unknown” in-vivo. Mucus flow rates (volumes) were based on observations from patients with post-TT otorrhea. Rates of TT occlusion were measured over a wide range of mucus flow rates. Higher flow rates (e.g., 1 ml / hour) resulted in nearly universal occlusion rates regardless of tube length, composition, and diameter. At much lower flow rates (i.e., 50 µl / hour) TT occlusion was not seen. Mucus delivery was increased until a consistent mid-range of occlusion was achieved with Goode “T” TTs (i.e., 50% occlusion of TTs over 2.5 hours with egg white delivered at 80 µl / hour or roughly 1 ½ drops / hour, total volume delivered was 0.22ml).

Mucus delivery was achieved using a custom built syringe pump consisting of a 5 led unipolar stepper motor driver kit (Jameco Diy Kit 109 Stepper Motor, 139109, Jameco Electronics, Belmont, CA) attached to a 24V stepper motor (Sanyo, 15837 MS, www.MPJA.com). The rate of mucus injection into the chamber was half that of the air injection (every 4 minutes) and was modeled to occur as a bolus, such that a fraction of the total mucus volume was delivered at each episode. This system allowed for computer control of the mucus delivery rate.

Air Delivery

Air flow was modeled to occur as a bolus, such as occurs with swallowing, nose-blowing, and sneezing. Air was delivered to the ear chamber via an air compressor which was controlled with a pressure regulator and a solenoid valve (Clippard, EVO-3, 12VDC, Cincinnati OH). A T-valve was used to divert part of the air flow to a pressure transducer (Validyne, DP15-46, 1546N1S4A, Northridge, CA) before entering the chamber (Figure 3-2F&H). The pressure

transducer was then connected to a digital-to-analog signal conversion board (Measurement Computing, PMD-1208FS, Norton, MA). Flow was controlled and pressures recorded using commercially available software (LabView version 6, National Instruments, Austin TX).

Boluses of air were delivered every other minute, corresponding to normal human swallowing [42]. Air was delivered at a volume twice that of hourly mucus flow. Air pressure was determined by titration in pilot experiments. Pressures set to 25 mm H_2O resulted in 100% occlusion rate, independent of TT design. Pressures were gradually increased until the occlusion rate decreased to 50%. This pressure value was (~ 110 mm H_2O ±20mm H_2O) and served as the standard pressure for comparing TTs.

Mucus Analog Preparation

A human mucus analog consisting of chicken egg white (albumin) was used. Egg white was chosen due to its very stretchy (stringy), non-homogeneous viscous nature (~4500cp non-homogenized) [43] along with its ability to form a solid crust-like material after drying, similar to human mucus [44]. These characteristics facilitate rapid experimental analysis, providing occlusion results within a matter of hours as opposed to months for in-vivo experiments. The egg white was carefully separated from the yolk, homogenized using a long thin blade, and stored at 5°C until use. Egg white contaminated with yolk was discarded. The resulting egg white samples had a physical consistency of serous mucus [44] and a viscosity of 388 ± 5.8cp, similar to homogenized human mucus (402 ± 9.1cp). For each experiment a one ml syringe was filled from the plunger port with 0.3 ml of albumin to prevent bubbles and reduce waste.

Tympanostomy Tube Testing Protocol

TTs of each design were inserted into latex membranes and tested in a random sequence. A temperature controlled chamber was placed over the testing device and maintained a constant 37°C environment. The chamber was pre-filled with 0.02-0.03ml of egg white. The chamber

pressure was recorded every second and stored in an Excel (Microsoft, Redmond, WA) file. Raw pressure data were compiled and normalized. A plug was defined as a peak in the chamber pressure equal to the input pressure (~110 cm H_2O). Pressure spikes below this input pressure were considered partial occlusions.

Zebrafish Preparation and Housing

Zebrafish were obtained from the Aquatic Toxicology Facility at the Center for Environmental and Human Toxicology (Building 118 Mowry Road). Fish were transferred at 4 dpf (days post fertilization) into 24 well plates (~10 fish per well). The fish were maintained in embryo medium (13.7 mM NaCl, 540 µM KCl, pH 7.4, 25µM Na_2HPO_4, 44µM KH_2PO_4, 300µM $CaCl_2$, 100µM $MgSO_4$, 420 µM $NaHCO_3$, pH 7.4) at 28.5°C [29]. After hatching, 5dpf fish were exposed to dissolved and filtered gel tubes. Following exposure, a 1mM DASPEI fluorescent dye solution was created using embryo medium and the larvae were incubated within this solution for 15 minutes. After exposure to the fluorescent dye, the fish were washed in embryo medium and anaesthetized with 10µg/ml MESAB (0.5mM 3-amino-benzoic acid ethyl ester, 2mM Na_2HPO_4). Following this, the fluorescent intensity, neuromast area, and number of viable cells were measured. After experimentation, the fish were euthanized using MESAB (250mg/L MESAB buffered with 200mg $NaHCO_3$/L) for 5 minutes or until all respiration had ceased [28].

Power Analysis: The number of fish required to achieve significant results for experimentation was determined by performing a power analysis. The power analysis was completed by consulting previously published work concerning the assessment of ototoxic and otoprotective agents [29]. Using results obtained from their research a delta of 1.0 was detected with a power of 0.8, an alpha level of 0.05, and a sample size of n=17 was obtained using

Minitab (Minitab 13 Statistical Analysis Software). The value of delta constitutes a modest change in toxicity that is both biologically, as well as statistically significant.

Statistical Analysis

Statistical analysis was conducted on all experimental results using a one-way un-stacked ANOVA test (Minitab 13, Statistical Analysis Software Package). P-values below 0.05 were considered statistically significant (95% confidence interval).

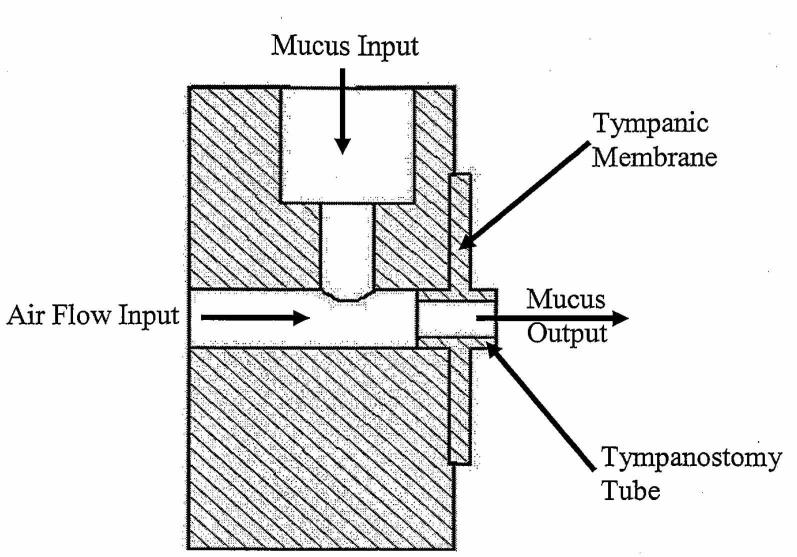


Figure 3-1: Schematic overview of the ear flow chamber.

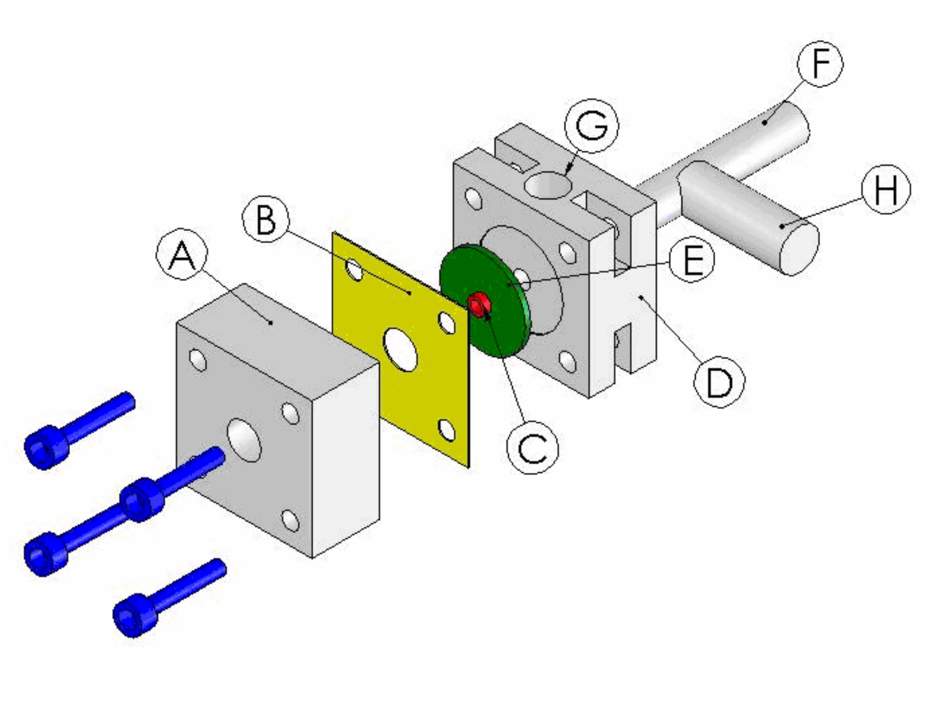


Figure 3-2: Exploded view of the in-vitro ear chamber. (A) Front compression plate. (B) Foam seal. (C) Vent tube. (D) Ear chamber. (E) Latex membrane (diaphragm) with ring. (F) Input for air. (G) Input for mucus. (H) fenestration for measuring chamber pressure.

CHAPTER 4
RESEARCH DESIGN AND METHODS

Specific Aim 1: Develop an Alginate Gel Tympanostomy Tube

Rationale

TTs are described as any flanged tube manufactured out of biocompatible components that is capable of providing reliable ventilation and limiting the development of secondary complications. Critical parameters involved in developing a viable ear tube are mechanical stability and consistent ventilation (low occlusion rate). When retained, commercial tubes are surgically removed and are incapable of dissolving in-situ with the use of non-ototoxic techniques. On the other hand, alginate TTs can be developed using biocompatible/dissolvable ingredients, providing a non-toxic non-surgical removal method. Mechanical strength can also be optimized to meet or exceed that of commercial tubes. Furthermore, design modifications can be directly incorporated into the alginate gels limiting the occlusion rate.

Alginate was chosen as the tube material due to its unique ability to rapidly cure and its high biocompatibility within the body [45]. Furthermore, alginate has low cytotoxic and hemolysis effects. Alginate gel can also be inertly crosslinked into a solid calcium-alginate gel with the use of calcium chloride (CaCl) and dissolved back into solution with the addition of sodium (Na). Moreover, alginate can conform into any shape allowing it to be molded into various tube diameters and geometries. In addition, alginate is an abundant substance in the food industry and is available in large quantities, making it inexpensive to produce.

Parameters Involved in Developing a Viable TT

The development of a reliable tympanostomy tube includes 4 main parameters: mechanical strength, geometry, dissolution, and biocompatibility.

Mechanical Strength: Maintaining a compressive strength above that of commercial tubes provides an environment for increased ventilation performance. Exposure to ototopical solutions/in-vivo environments can decrease mechanical strength (ventilation performance) leading to an increased incidence in occlusion formation. Parameters that may affect gel strength include alginate/PEG ratio, Ca concentration, and desiccation time.

Geometry: Tube geometry, specifically barrel surface roughness, can contribute to increased occlusion rates. Alginate surface roughness can be minimized using topical treatments. Parameters that may affect smoothness include manufacturing techniques, and albumin coating.

Dissolution: Commercial tubes that become retained require invasive surgical removal procedures. However, alginate tubes have the ability to dissolve in-situ using minimally invasive techniques. Parameters that may affect dissolution include alginate/PEG ratio, Ca concentration, desiccation, dissolution solution concentration, and dissolution additives.

Biocompatibility: Biocompatibility associated with alginate tube dissolution reduces patient trauma and provides an environment for healing. Parameters that may affect biocompatibility include PEG concentration, Ca concentration, dissolution solution concentration, and dissolution additives. Each of the above parameters was investigated and optimized in order to develop a reliable tympanostomy tube.

TT Design

A negative aluminum mold was machined to produce several types of tubes (Figure 4-1) (2mm long x 2mm wide bobbin-tubes and 6mm long x 3mm wide T-tubes). The bobbin- tubes have an inner diameter of 1.02mm and the T-tubes have an inner diameter of 1.14mm. T-tubes were used in testing mechanical strength (tension and compression) while Reuter Bobbin tubes were used in testing occlusion rates. The inner lumen of the tube was developed using a glass

rod (a) which provided a smooth crosslinking surface and aided in tube removal. Depressions (b) on one/both sides of the mold created the tube flanges. Final tube dimensions were increased ~80% to account for shrinkage during drying. The barrel wall thickness, after drying, was set at ~0.1-0.2mm.

Tube construction consisted of injecting Alginate:PEG (< 0.1ml) solution into the mold followed by the application of CaCl along the top flange surface of the gel. Twenty to thirty minutes later, additional CaCl solution was injected through the top flange of the gel and into the bottom (barrel) portion of the mold, curing the remaining alginate gel. Thirty minutes later, (total curing time ~60 minutes) the T-tube was removed by pulling the glass rod and gel out through the top of the mold.

Reuter Bobbin tubes were created by injecting Alginate:PEG (< 0.1ml) solution into each mold using a 25.5 gage syringe with a 45° bend at the end. Following this, CaCl (< 0.1ml) was injected using a 27.5 gage syringe with a 90° bend. The syringe needles were bent in order to completely inject the solutions into the bottom "hidden" flange of the mold. The CaCl was injected first into the bottom flange of the mold followed by additional CaCl solution along the top surface of the mold facilitating curing of both the flanges and barrel. After 45-60 minutes (total curing time) the mold was separated gaining access to both flanges. Using tweezers, one flange was pushed through the opening of the form and out of the mold. Care was taken to prevent the glass rod from falling out during tube extraction. During extraction one of the two flanges was damaged requiring repair. The damaged flange was rendered functional after manipulation and application of additional CaCl curing solution.

Tube drying

Fresh alginate gel tubes were prepared for implantation via desiccation. Fresh tubes were very flexible, large, and soft making them difficult to manipulate for implantation. Desiccation removes water and dries the tube, potentially increasing strength, while preventing additional shrinkage after implantation. Prior to drying, 0.01ml CaCl solution was applied to the gel tubes prolonging desiccation time. Measured, deliberate drying first at 23°C (24 hours) followed by 7-10 days at 37°C, removed water from the gel matrix, prevented additional shrinkage after implantation, and produced a rigid strong tube. Tubes were suspended using the glass rod over the wells of a 24 well plate preventing the flanges from flattening. After drying, tubes were cut to a specific length and slid off their glass supports. Glass rods were removed prior to experimentation and were used to position the tube for tympanic insertion.

Mechanical Testing

The mechanical properties of the alginate gels (T-tube configuration) were measured by quantifying the modulus of elasticity and compressive strength of alginate gel according to ASTM standards (D 638, D 882, D 3575, F 2064) [38,46-48]. These results were compared with those obtained from commercially available silicone tubes. The silicone tube (Goode T-tube, Silicone, 1.14mm ID, 12mm length, Medtronic-Xomed Inc. Jacksonville FL) was used as a benchmark for comparison with alginate gel tubes due to its relatively low mechanical strength and widespread use.

Tension testing

Samples of alginate gel were molded into "dog-bone" specimens, dried, and clamped at either end to a support (n=10 for each concentration). A dog-bone mold was cut into a 2.5mm thick PDMS plate using an x-acto knife placed over a dog-bone pattern. Each specimen was cast to a width of 2.0mm (at the thinnest section) and a length of 20mm. Alginate:PEG solution was injected into the molds followed by CaCl curing solution. Glass microscope slides were placed

over the curing gel to prevent gel movement. Gels were cured for 30 minutes, removed from the mold and additional CaCl curing solution (0.1ml) was added to the gels (groups of 5) to prolong desiccation and promote crosslinking. Gels were kept in the incubator until testing. The dry gel thickness and width were measured using a digital caliber (Mitutoyo Corp, 500-196, CD-6"CS). Silicone samples were cut into "dog-bone" specimens from existing silicone tubes (12mm long, 0.25mm thick, center section width of 1-3mm, and an overall width of 5mm).

Samples were loaded into a mechanical loading device (Figure 4-2, 4-3) (Appendix A) and a camera system was used to record the deformation against a calibration chart. Each specimen was placed into the tension grips (Figure 4-2B) starting with the top grip, then lowered into the bottom grip. Soft foam inserts were glued to the insides of the aluminum clasps increasing friction and preventing gel damage. Samples were pulled with a constant strain rate using a manual screw drive (Figure 4-2C) while a load cell (Honeywell-Sensotech, 34, 5 lbs, Columbus OH), attached to the bottom grip, recorded the stress on the sample. The control arm (slide) Figure 4-2D prevented rotation of the sample during loading. A Labview (Labview National Instruments v8.0, Austin TX) data acquisition system along with a DAQ board (Measurement Computing, PMD – 1208FS, Norton MA) recorded and analyzed the results to determine the modulus according to Hooke's Law Equation 4-1. Prior to experimentation samples were pre-loaded with ~5-10 grams (0.022 pounds) preceding stretching. The alginate gel samples used for mechanical testing were assumed to be isotropic.

$$E = \frac{\sigma}{\varepsilon} \tag{4-1}$$

E= Young's Modulus, σ = stress (Force/Area), ε = strain ($\Delta L/L$), L = length of gel sample, Area = cross-sectional area

Data analysis

Video from each sample was digitized and opened (7 in long by 6 in high) in QuickTime (QuickTime, Apple Inc.). The corresponding Excel file, obtained from the Labview program, was also opened and graphed. The video was then cued up to the frame prior to failure. This frame corresponded to the point of maximum tension on the Excel file and was used as the starting point for analysis. The conversion factor between the screen image and actual dimension was determined. Stress/Strain values were obtained every second (corresponding to 10 data points (data acquisition 10 Hz)) and recorded on the excel sheet starting from the max load and decreasing until the load measured 0g. This entire procedure was repeated for the each of the remaining tension experiments.

Tube compression

Gel samples were also manufactured in a tube configuration and their compressive strength was compared with commercially available silicone tubes. During the healing process, the skin of the tympanic membrane compresses the barrel of the tube and in many cases can extrude it. However, if the tube is too weak (mechanically), then the encroaching skin will compress the barrel of the tube and occlude it. Therefore, the amount of force required to compress the barrel of the alginate tube was measured and compared with the commercial silicone tube.

Compression testing consisted of deforming the barrel of the tube between two flat platens while a camera system (Sony, ExwaveHAD Color Video Camera, DXC-190) attached to a zoom lens (Edmund Industrial Optics, VZM 450i) observed the distance between the top and bottom platen. The top platen was composed of aluminum, while the bottom platen was composed of PDMS. A funnel composed of paper was attached to the aluminum platen and extended up into a set of support arms providing stability (Figure 4-4). Prior to testing, part of the T-tube flange was removed to allow the gel to lie flat on the testing surface. Red dye was applied to the front

surface of the tube increasing visibility (normally the gel tube is clear). Tube symmetry was maintained after coloring with the re-insertion of the glass support rod. A calibration ruler was placed on the video screen and initial measurements were taken of the diameter of the tube and its placement on the bottom platen. The tube was not moved from this point on. The top platen with the funnel was then carefully placed on top of the tube (1mm from front edge) and adjustments were made to align the ruler on the video screen. Sugar was then slowly added to the funnel while the distance between the platens was monitored on the screen (Appendix A, Figure A2. After compression (25%), the funnel (sugar, top platen, and funnel) was removed from the tube and weighed. The compression strength [46] of the tube was obtained from Equation 4-2 below.

$$CD = \frac{F}{A} \tag{4-2}$$

CD = compression deflection (compressive strength) force per unit of specimen area, (kPa). F = force required to compress the specimen 25% outer diameter, (N). A = specimen compression contact surface area, (m^2).

Data analysis

The contact surface area (A) from the above equation was calculated using the conservation of volume and Equation 4-3 (undeformed condition) and Equation 4-4 (deformed condition) after 25% compression (Figure 4-5). The mechanical properties of the alginate tubes were modified based on the ingredient concentrations while maintaining uniform dimensions (inner diameter, outer diameter, wall thickness) across all tube types. As a result, only the force (F) required to deform each of the tubes (25%) will differ. Therefore, assuming that the contact area of each tube was uniform along with uniform wall thickness (h = constant before and after deformation) the contact area (2b) can be determined by setting the front surface area of the

tubes equal to each other (Equation 4-3 equal to the top portion of Equation 4-4) and solving for b (Equation 4-4 bottom). In 3D the contact area was described as a rectangle with sides equal to 2b and a length of L (tube length) (Appendix B). Each experiment was performed 10 times to ensure accuracy.

$$Area(A_o) = A_2 - A_1 = \pi r_2^2 - \pi r_1^2 \tag{4-3}$$

$$\begin{aligned} Area(A_o) &= 2(2b \times h) + 2\left(\frac{\pi r_{2b}^2}{2} - \frac{\pi r_{1b}^2}{2}\right) \\ r_{1b} &= r_{2b} - h \\ r_{2b} &= \frac{1}{2}(0.75)(d) \\ h &= thickness\ (constant) \\ d &= tube\ \ outer\ \ diameter \\ b &= \frac{A_o - \pi(r_{2b}^2 - r_{1b}^2)}{4h} \end{aligned} \tag{4-4}$$

Maximize Mechanical Strength

Using the methods described above, the mechanical properties of the gel tubes were altered by changing the alginate/PEG ratio, Ca concentration, and desiccation time. Unless otherwise noted, tubes were dried for 7 days prior to testing. Each parameter was tested separately via tension and compression experiments to isolate their effect on the mechanical strength. Tension and compression results were compared to commercial silicone Goode T-tubes.

Alginate:PEG concentration

The mechanical strength of the gel was enhanced by increasing the alginate ratio within the gel. Alginate bonds with Ca from the crosslinking solution and creates a solid gel. Therefore, the more alginate present in solution the more bonds will be created. PEG promotes dissolution by limiting the amount of alginate available for crosslinking. Gel samples with Alginate:PEG ratios (3% Alginate, 0.001M PEG determined from literature) ranging from 4:1 to 2:1 and finally

1:2 were created and mechanically tested. The mechanical strength of each gel configuration was determined via tension and compression experiments. Initial experiments were crosslinked with 1.0M CaCl solution.

Ca concentration

In addition to the Alginate:PEG concentration, the Ca concentration was maximized to promote mechanical strength. Higher quantities of Ca within alginate gels have been shown to make stronger gels [49]. Therefore, the concentration of Ca within the curing solution was increased from 0.5M to 1.0M and finally 2.0M. The calcium concentration range was determined from previous studies [13,40]. The mechanical strength of each gel configuration was determined via tension and compression experiments. The Alginate:PEG ratio used for testing was determined from the above Alginate:PEG concentration experiments.

Drying time

As the gel tubes dry, moisture was removed, increasing rigidity. The effect of moisture loss on gel tube strength was measured after drying over several days. Prior to desiccation "fresh" tubes contain the highest concentration of water and are mechanically weak. Therefore, desiccation time (37°C incubator) was increased, while measuring the mechanical strength, from 1, 2, 3, 4, 5, 15, and 30 days, holding the Alginate:PEG ratio and CaCl concentration constant (max values) from the above experiments.

Physiological Testing

The above experiments were designed to quantify mechanical strength based on tension and compression experiments. Additional experimentation was conducted on samples subjected to physiological conditions including agar encapsulation, ototopical solution exposure, and tube plugging. These conditions simulated environments that may be encountered during in-vivo implantation.

Quantify mechanical strength after agar encapsulation

TTs are designed to be implanted within a warm moist environment (tympanic membrane) for several months until clinical symptoms resolve. During implantation it is possible for the tubes to become encapsulated by the surrounding tissue. In-vivo tissue encapsulation was simulated by encapsulation within an in-vitro agar substrate (30% w/v Pluronic F-127 added to 125ml 0.85% NaCl, Sigma P2443). The solution was allowed to rest for 3 days at 5°C until homogeneous, followed by autoclaving for 15 minutes at 250°F. After cooling, (5°C) the agar solution was mixed with antibiotics (Antibiotic Antimycotic Solution, A9909, Sigma) in a 10:1 ratio (agar : antibiotic) and poured into culture plates. Pluronic solution is unique in that at 37°C the gel forms a solid, while at 5°C the gel is a liquid, making tube insertion and extraction straightforward. After submersion for 15 and 30 days, tubes were removed from the agar, washed in phosphate buffered saline (PBS) several times to remove remaining surface agar, and compression tests were conducted. Results were measured and compared to both a non-encapsulated alginate control and silicone commercial tube.

Quantify mechanical strength from exposure to ototopical solutions

During treatment, solutions may interact with the TT that can become detrimental, prematurely degrading the gel and limiting ventilation. These ototopical solutions include pool water, soapy water, salt water, hydrogen peroxide, blood serum, vinegar, ear mucus, and antibiotic treatments or ear drops (Ciprodex, Ofloxacin). Children are the primary benefactors of TT placement and periodic exposure to the above solutions is possible. Therefore, tubes created using the strongest ingredient ratios (determined from above experiments) were submerged in the above solutions for 24 hours, at 37°C.

Solutions were prepared as follows. Chlorinated pool water (1-3 ppm) was applied (0.5ml) to dried alginate gel tubes and the mechanical strength was recorded. Furthermore, soapy water (hand soap (0.5ml)), salt water (35g Na/kg water), hydrogen peroxide 3%, vinegar (1:1 dilution), human ear mucus (pooled samples), as well as FDA approved over the counter ear drops (0.5ml) (Ciprodex, Ofloxacin) [26] were also exposed to the gel tubes. In addition, blood serum 0.5ml was added to the gel tube enabling measurement of the effects wound healing might have on mechanical strength. Serum was collected by centrifuging whole blood at 1,500rpm for 10-15 minutes followed by aspiration of the top "clear" serum layer above the red blood cell layer. The concentration of salt water (35 g Na/kg water) was chosen to be similar to the sodium concentration present in ocean sea water. Alginate gel samples remaining intact after submersion for 24 hours were mechanically tested (tube compression). Results were compared to a no-treatment control as well as commercial silicone tubes.

Quantify plugging of the alginate gel tube

The performance of the above ear tube was further characterized by comparing the plugging frequency of the reuter-bobbin-shaped alginate tube (1.02mm ID, 2 mm long, alginate, with and without human serum albumin) to a commercial stainless steel reuter-bobbin tube (1.02mm ID, 2 mm long, stainless steel). Among commercial tubes, stainless steel TTs have the highest occlusion rate and were therefore chosen for comparison with alginate tubes. Successful gel tube designs should minimize plugging at or below commercial levels.

Plugging was minimized with the application of albumin (0.01% Human Serum Albumin (HSA) (Sigma, A6909 30% in 0.85 NaCl, diluted in phosphate buffered saline (PBS))) along the surface of the tube. Albumin has been shown to decrease surface roughness and limit occlusion [7]. Albumin application was achieved after removal of the Alginate TTs from their glass support

rods and submersion in the albumin solution overnight at room temperature. Tubes were then rinsed (2 times) in 10mM phosphate buffered saline (PBS) and dried with a Kim-wipe. Glass rods were re-inserted after HSA application to reform the tube lumen. Gel tube samples were tested, with and without human serum albumin, against commercial stainless steel tubes and in-vivo clinical trials. Uncoated alginate tubes were submerged overnight in PBS only (no HSA). All experiments were performed using an in-vitro ear chamber [50] outlined in Chapter 3. The strongest tube composition as determined from mechanical testing experiments (Alginate:PEG ratio, CaCl concentration, drying time) were developed into Reuter Bobbin tubes and tested against the stainless steel tube. Stainless steel commercial tubes were not coated with albumin. Comparisons were made between un-coated alginate tubes and un-coated commercial stainless steel tubes as well as with albumin coated alginate tubes. Plug formation was measured by comparing the experimental pressure to the known input pressure. Occluded tubes have the same pressure reading as the input pressure. Each experiment was performed 10 times.

Specific Aim 2: Optimize the Gel Dissolution Components for Complete Dissolution

Rationale

Dissolvable TTs provide a minimally invasive method for removing ventilation tubes. Currently, retained commercial tubes are surgically removed leaving the patient with an increased risk of complications and trauma. Absorbable tubes have been developed to eliminate or at least reduce the need to surgically remove displaced tubes. However, their rate of dissolution, in-vivo, cannot be controlled. Once implanted, absorbable tubes have a pre-set resorption time.

On the other hand, dissolvable ear tubes composed of calcium/alginate/PEG have the potential to dissolve only in the presence of a dissolution solution (ex. sodium bicarbonate ($NaHCO_3$), NaCl, or vinegar), providing uninterrupted function if additional complications arise.

These solutions were chosen as potential dissolution solutions due to the presence of Na ($NaHCO_3$, NaCl), one of the ions capable of dissolving alginate, as well as their use in previous studies treating TT sequelae (complications).

Since mechanical strength is directly related to tube ventilation, priority was given to mechanically superior tubes over rapidly dissolving ones. Altering mechanical strength may facilitate rapid dissolution, but at the cost of functionality. Therefore, dissolution was optimized with regard to dissolution additives and dissolution concentration only. (If not otherwise stated the gel tube composition (T-tube shape) for the following experiments was 3% Alginate in a 4:1 ratio with 0.001M PEG, 0.5M CaCl, and 0.5M $NaHCO_3$ as the dissolution solution.)

Dissolution Analysis

Image analysis

Dissolution images were obtained using a CCD camera (Hamamatsu, C2400) attached to a macro lens (Sigma Macro f=50mm 1:2.8). Images were taken at 0, 1, 2, 5 hours. Photographs were adjusted for light intensity (Microsoft Word, Format Picture (Brightness-Contrast)) to highlight the dissolution products.

Dissolution rubric

Tubes were prepared for dissolution after removal from their glass support rods and placed within a 24 well plate. Images were taken prior to dissolution at t=0 hours and again at 1, 2, and 5 hours. Dissolution rate was determined using a dissolution rubric (R value) (Table 3). The rubric consists of 6 stages: R=1 (NO dissolution) through R=6 (complete dissolution). Images were adjusted for light intensity bringing out the color and contrast of the gel products in solution. R-value averages were compiled and graphed.

Optimize the Dissolution Components for Complete Dissolution

Dissolution additives

Ototopical solutions, including sodium chloride, vinegar and $NaHCO_3$, have been used clinically to treat TT sequelae and may potentially accelerate tube dissolution. Sodium (Na) within the NaCl and $NaHCO_3$ may degrade alginate TTs by replacing Ca within the crosslinked gel. Vinegar along with sodium bicarbonate is highly reactive producing carbon dioxide gas which may also be used to degrade alginate gels. Tubes were exposed to 0.5ml of sodium chloride (0.85%) along with 0.5ml of vinegar (1:1 dilution, household distilled) and $NaHCO_3$ [1.0M]. In addition, 50/50 ratios were created of NaCl and vinegar with sodium bicarbonate solution.

A range of tube compositions, consisting of high and low concentrations, were developed based on the results from Specific Aim 1. Priority was given to those solutions that dissolved mechanically superior tubes since mechanical strength was conserved over rapid dissolution. Tubes were tested against the above dissolution agents and those solutions providing complete dissolution (R-value >3) within 1-5 hours were used in further dissolution experiments.

Dissolution concentration

Adjusting the quantity of Na present in solution may regulate the rate of alginate dissolution. The optimal Na concentration designed to minimize dissolution time was obtained by increasing the concentration of dissolution solution (ex. $NaHCO_3$) from 0.1M, to 0.5M, 1M, and 2M. Results were quantified using the rubric in Table 3.

Physiological Testing

Above experiments were dissolved by complete submersion within the various dissolution solutions. However, in-vivo, dissolution solution is unable to completely envelop the tube due to

physiological limitations. Therefore, a dissolution chamber was created to simulate in-vivo dissolution conditions.

Dissolution chamber

After determining the optimal dissolution solution and concentration from the above tests, the remaining experiments were dissolved within an in-vitro ear chamber capable of simulating the in-vivo tube conditions. After in-vivo implantation, access to the tube for dissolution was limited. Therefore, an ear chamber was designed that allowed dissolution solution to be applied only to one side of the tube (as seen in-vivo). Tubes (T-shaped) were suspended within a latex membrane (flange side out) and supported by a thick metal ring. The thickness of the metal ring (3mm) above the latex simulated the external ear canal and provided a holding area for the dissolution solution. The barrel portion of the tube was suspended over a 24 well plate representing the middle ear space. This implantation method differs (inverse) from that of in-vivo implantation where the flange portion of the tube is implanted facing the middle ear space and the barrel portion is implanted facing the external ear canal. Tube implantation was modified in order to simulate a “worst-case” scenario. Access to the thicker flange portion of the tube during removal (rather then thinner barrel portion) prolonged dissolution and represented the maximum time frame (worst case) necessary for tube removal. Furthermore, mounting tubes using this inverse method minimized tube extrusion during dissolution. Allowing the flange to face the external ear canal prevents the gel tube from extruding from the latex membrane. The tubes were suspended vertically allowing solution to flow through the tube. T-shaped tubes were chosen due to their larger size (worst-case) and ease of manufacture. 0.5ml of dissolution solution was applied to the external ear canal and the tubes were dissolved from the flange side to the barrel side. Dissolution times were recorded and compared among samples.

Quantify the effect of dehydration on the dissolution rate of the gel tube

Throughout implantation, moisture was continuously removed and the tubes became increasingly rigid. Increased mechanical strength and decreased dissolution may result from increased rigidity. As a result, tubes were dried for 0, 15, 30 days at 37°C [51] and the dissolution rate was compared. Dissolution was performed within the in-vitro ear chamber.

Quantify the effects of human serum albumin coating on dissolution

Coating tubes with 0.01% human serum albumin (HSA) (Sigma, A6909, diluted in phosphate buffered saline (PBS)) reduces the occlusion rate and limits patient morbidity [7]. However, HSA coatings may alter the dissolution rate of the gel after application. Therefore, HSA coatings were applied to alginate tubes overnight and dissolved using the in-vitro ear chamber. R-values were obtained after 1-2 hours and compared to non-coated (PBS only) alginate controls.

Specific Aim #3: Quantify Ototoxicity from Alginate Gel Dissolution using Zebrafish: Rationale

Minimizing the ototoxicity associated with alginate gel tube implantation and especially dissolution is critical to the acceptance of dissolvable alginate tubes as a viable alternative to commercial tubes. The purpose of these ototoxicity experiments was to measure and quantify toxicity associated with the dissolution of the alginate gel tubes. High toxicity can lead to patient trauma including permanent hearing loss. Ototoxicity can be measured using an established Zebrafish model. The resulting fluorescent intensity is directly related to hair cell ototoxicity and can be compared to known ototoxic agents (Cisplatin). Viable hair cells within the neuromasts of Zebrafish uptake fluorescent dye and glow. Non-viable cells do not uptake the dye. This assay provided a quantitative analysis of the ototoxic effect of alginate gel tube dissolution.

Quantify Ototoxicity from Dissolution Products

Fluorescent Staining: Zebrafish were fluorescently stained with 1mM DASPEI solution made from embryo medium for 15 minutes. The Zebrafish were then rinsed in fresh embryo medium three times (5 minutes each), and anesthetized with 10μg/ml MS-222 for 15 minutes. Fish were then transferred using a transfer pipette to a microscope slide with a depression, and analyzed under a fluorescence microscope equipped with a DASPEI filter (450-490nm). Excess embryo media was siphoned from the microscope depression using a kim-wipe. Neuromast hair cells are located along the later line of the Zebrafish as well as along the head region (Figure 4-6) [28]. A fluorescent intensity map of the target neuromast (P4) was measured followed by specific localized analysis (neuromast area and number of hair cells labeled with DASPEI dye) (Figure 4-6). Fluorescence intensity is directly related to the number of hair cells present within the neuromast and is a function of the area stained using fluorescence. After experimentation Zebrafish were euthanized using 250mg/L MS-222 buffered with ~200mg/L $NaHCO_3$.

Pilot Study: Ototoxicity testing began by limiting morbidity/mortality associated with the dissolution concentration and treatment exposure duration. Zebrafish are a freshwater fish and are comfortably maintained at $NaHCO_3$ concentrations at or below 0.004M in embryo media (Appendix E). However, the $NaHCO_3$ concentrations used to dissolve alginate gels varied between 0.1M and 0.5M $NaHCO_3$. Therefore, a pilot study was conducted to determine the concentrations necessary to maintain life while providing measurable results. To this end, $NaHCO_3$ concentration was reduced along with the exposure duration (24hours, down to 1 hour, 30min, 15min, 10min, and 5min) in the hopes of achieving a non-toxic threshold. Five day old embryonic Zebrafish (n = 3) were exposed to 0.1ml of the dissolution products (alginate gel tubes dissolved in $NaHCO_3$ and filtered (0.22μm)). The concentrations of the solutions were prepared using embryo media as the “base” mixing solution. Analysis consisted of

morbidity/mortality outcomes associated with decreasing the concentrations and exposure times of the dissolution products.

Once a survival threshold was determined, a positive control consisting of 50µM - 1mM Cisplatin (Sigma-Aldrich, P4394) [30] and a negative control consisting of embryo media were used to calibrate the UV analysis. The positive control has a well documented history of Zebrafish hair cell ototoxicity [29,30] and provided a baseline reading for toxicity. Positive control dosage was determined empirically in a pilot study identifying high dosages of Cisplatin that caused hair cell death without mortality within relatively short time periods. The concentrations of the solutions were prepared using embryo media as the "base" mixing solution. Fish were individually separated into 96 wells plates and incubated at 28° C for testing. After treatment exposure, fish were removed from the solutions and allowed to rest in embryo media for 3 hours prior to fluorescent analysis.

From this, a survival and ototoxicity chart was created. Those concentrations and exposure durations yielding measurable ototoxicity results (noticeable comparisons between treatments) while maintaining survival were used for experimental analysis.

Experimental Protocol: After determining the threshold values for Cisplatin, $NaHCO_3$, and exposure duration, a power analysis was performed to determine sample size (n = 17) (Chapter 3). Five day old embryonic Zebrafish were then exposed to 0.1ml of the positive Cisplatin and negative embryo media control along with dissolved and filtered alginate tubes (calcium alginate gel tubes dissolved in $NaHCO_3$ and filtered (0.22µm)). Once again the concentrations of the solutions were prepared using embryo media as the "base" mixing solution (Appendix C). Fish were individually separated into 96 wells plates and incubated at 28° C during testing. After treatment exposure, fish were removed from the solutions and allowed to

rest in embryo media for 3 hours prior to fluorescence. Comparisons were made between the controls and samples. Based on the ototoxicity results, dissolution was re-calculated using the concentration and exposure times determined from the Zebrafish experiments.

Fluorescent analysis

Fluorescent intensity was measured using Adobe Photoshop (Adobe Photoshop v7.0, Adobe Systems Incorporated). Neuromast P4 was isolated from each image and a fluorescent intensity map was created. DASPEI stain glows green under fluorescent light. A range of individual pixels representing the full range of green fluorescence (low to high) was selected using the "color range" feature in Photoshop. After selection, the "histogram" feature was used to quantify the average "green" luminosity (intensity). Neuromast area (in pixels) was also determined via the histogram results. Pixel area was converted to nanometers via a micrometer calibration. Individual hair cells were represented by circular globes of green light within the neuromast and were counted microscopically.

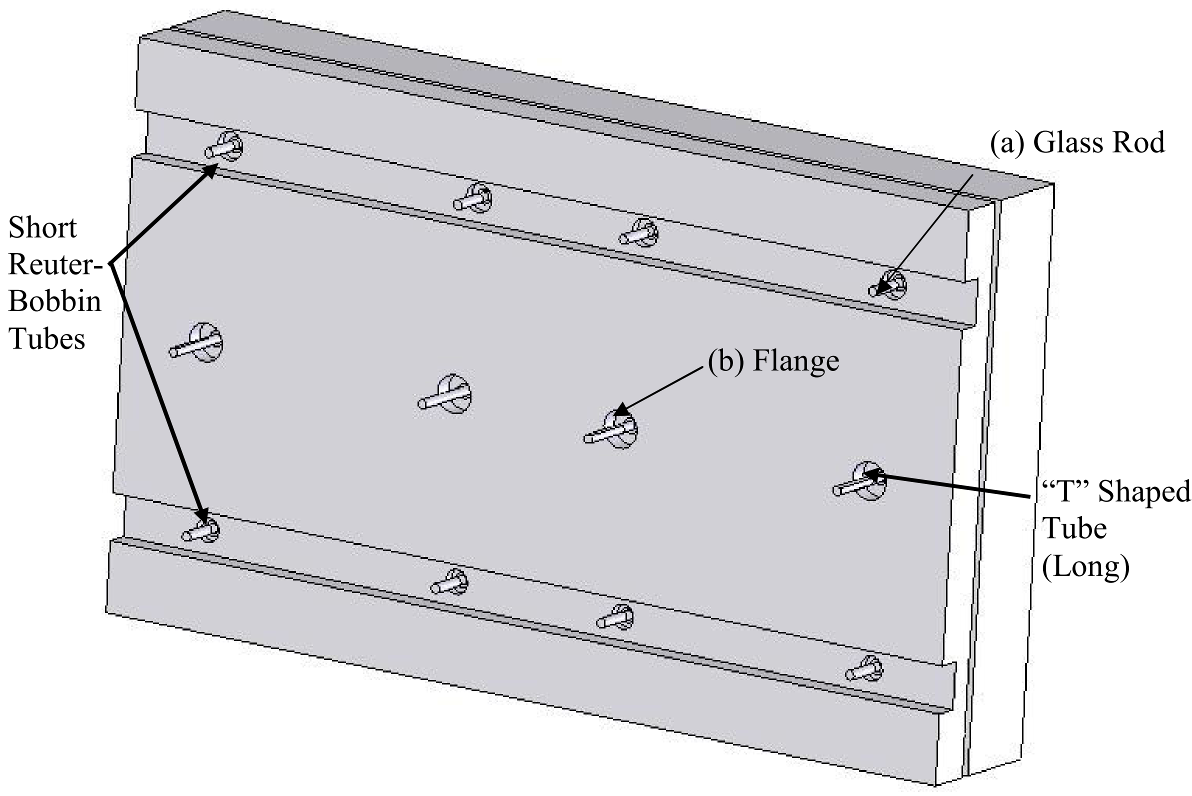


Figure 4-1: Aluminum ear tube mold used for creating bobbin and T-shaped tubes. (A) Glass rods. (B) Flange groove.

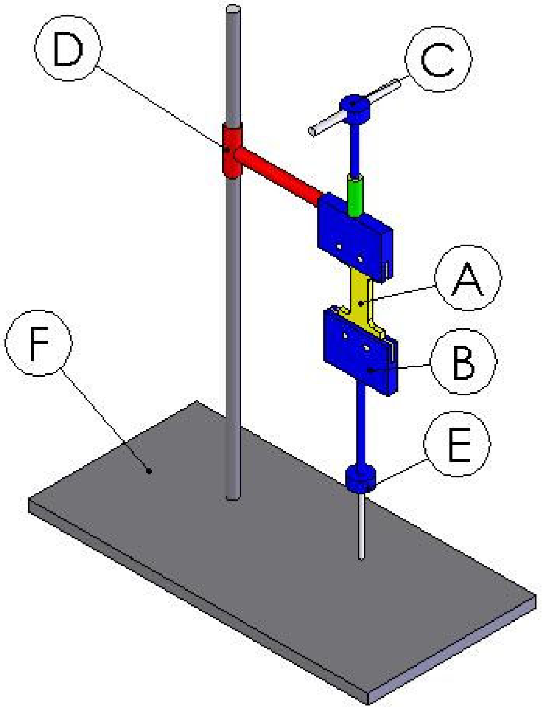


Figure 4-2: Mechanical testing device (Tension). (A) Dog-Bone testing specimen between clamps. (B) Sample clamps with foam inserts. (C) Hand screw drive. (D) Control arm (slide). (E) Load cell. (F) Stand base.

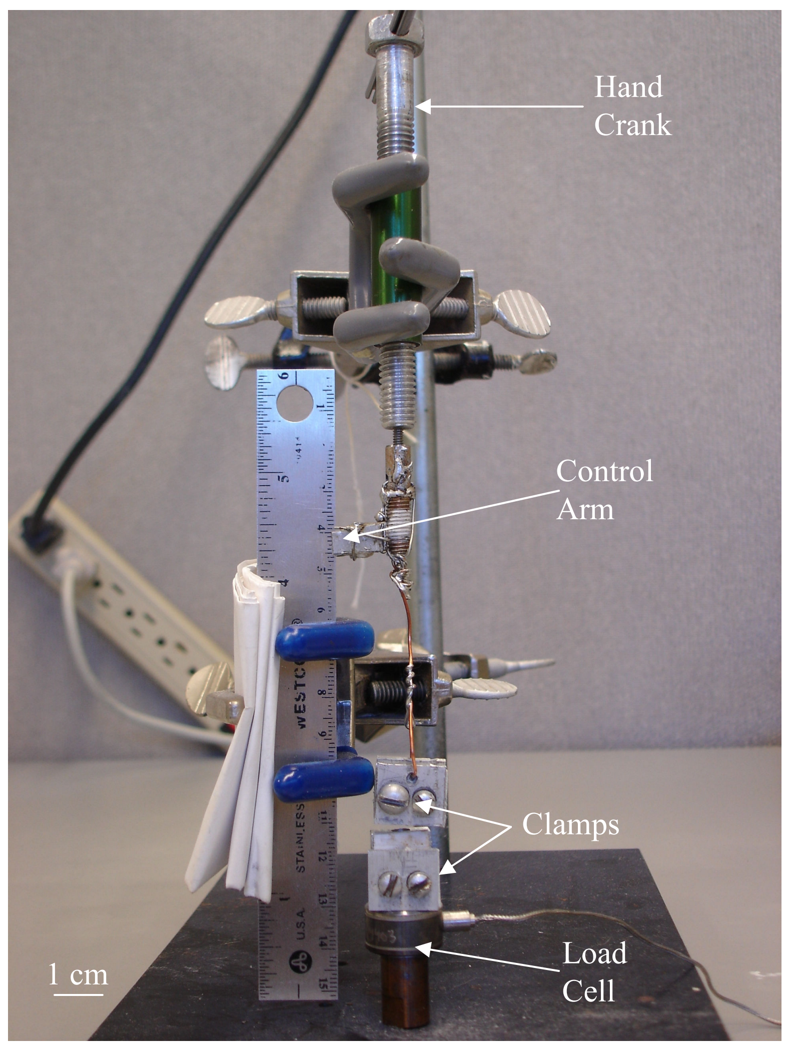


Figure 4-3: Tension testing apparatus setup.

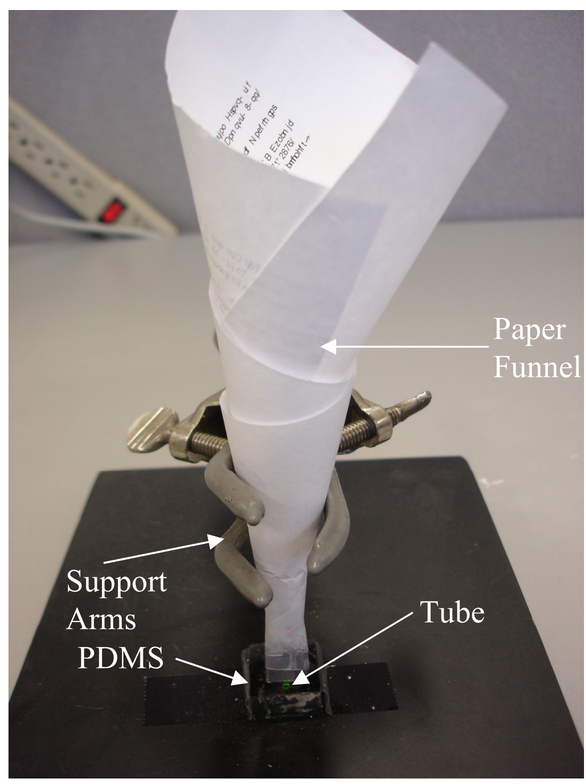


Figure 4-4: Tube compression apparatus.

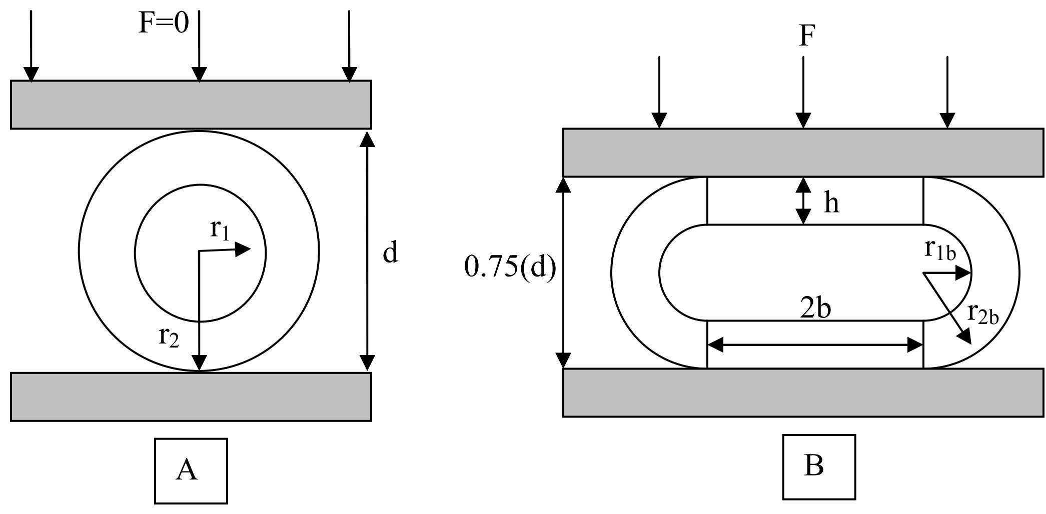
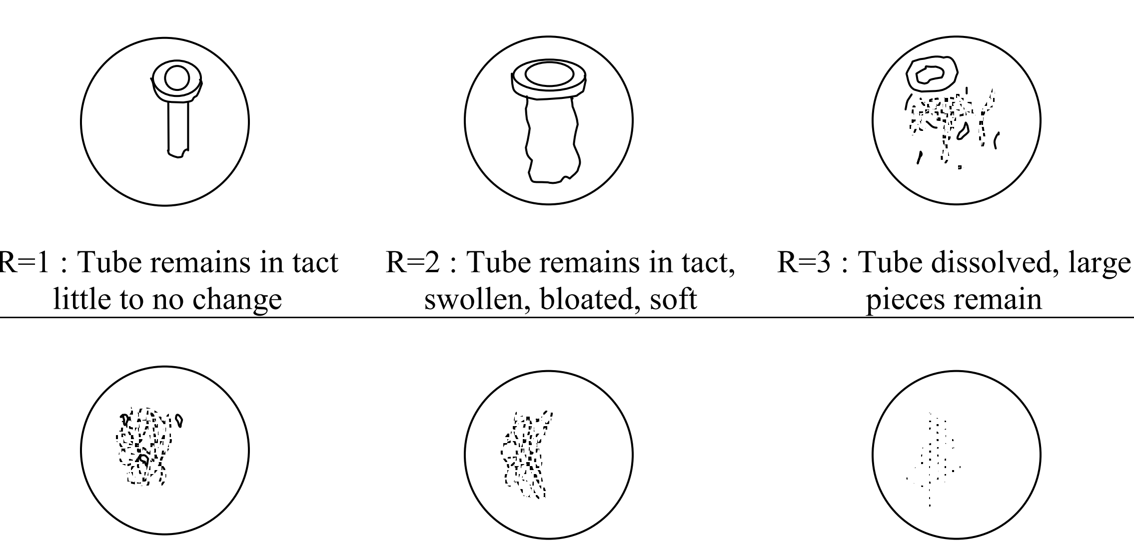


Figure 4-5: Tube compression contact area schematic. (A) Undeformed silicone and alginate gel tubes. d = distance between the two platens. d = outer diameter of tube. r_1 = inner radius. r_2 = outer radius. F = force (load) applied to the barrel of the tube. (B) Deformed tube (25%) showing contact area of 2b. h = tube wall thickness

Table 4-1: Dissolution rubric showing various stages of tube dissolution.



R=1 : Tube remains in tact little to no change	R=2 : Tube remains in tact, swollen, bloated, soft	R=3 : Tube dissolved, large pieces remain
R=4 : Tube dissolved, small pieces remain	R=5 : Tube dissolved, only fine particles remain	R=6 : Tube dissolved, tiny particles remain, clear

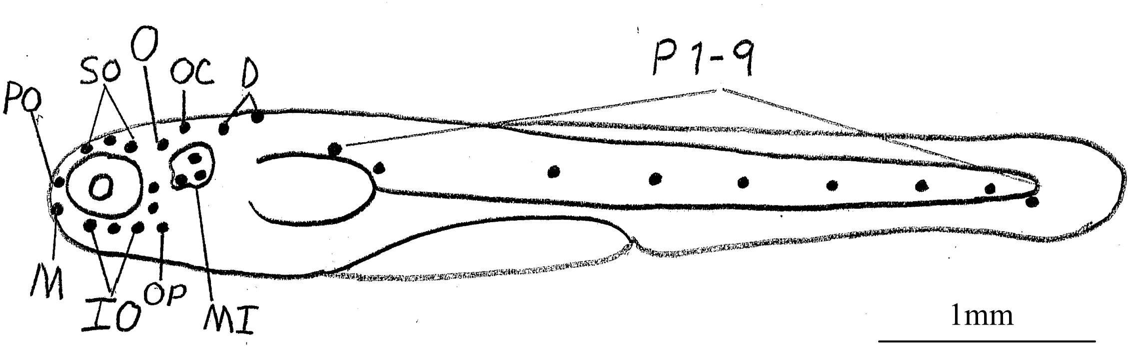


Figure 4-6: Position of head and lateral line neuromasts. Position names are based on body position. D = dorsal trunk, IO = infraorbital, M = mandibular, MI = middle, O = otic, OC = occipital, OP = opercular, P = posterior, PO = preoptic, SO = supraorbital.

CHAPTER 5
RESULTS

Specific Aim 1: Development of an Alginate Gel Tube

Both T-shaped and bobbin shaped alginate gel tubes were created using an aluminum mold. The results show that after 24 hours (at 23°C) the gel tubes had begun to dry and had retained most of their original size (90% original size). After 6 additional days (at 37°C) the gels were completely dried and could be moved freely on the glass rod. On the other hand, rapid desiccation (37°C for 5 days) proved to be detrimental, resulting in shriveled unusable tubes. Alginate T-tubes (Figure 5-1, 5-2, 5-3) have a flange on one end to prevent the tube from expiating after insertion. Alginate T-tubes have a dried barrel outer diameter = 1.5-1.6mm, a wall thickness = 0.1-0.2mm, a length = 5-6mm, and an inner diameter = 1.1-1.2mm. Reuter Bobbin tubes (Figure 5-4) have a flange on both sides with a short 1-2mm barrel between them. Reuter Bobbin tubes have a final dried barrel diameter of 1.6mm, a wall thickness of ~0.2mm, and a length of 2mm (Figure 5-5). Furthermore, dimensional constraints appear to be conserved after drying. However, dimensions can be altered depending on physician specifications and use.

Maximize Mechanical Strength (Tension)

Alginate:PEG Raito: Alginate gel strength was measured first in tension (dog-bone configuration) followed by compression (tube configuration) (See Appendix B). Dog-Bone specimens were created, dried for 7 days, and tested for each gel concentration and ratio starting with Alginate:PEG [4:1], [2,1], [1,2] crosslinked with [1.0M] CaCl solution. Experimental results show ratios lower than 1:2 (ex. 1:3, 1:4…) fail to produce solid gel tubes. The dried dog-bone dimensions were 10-12mm long, 1-2mm wide and 0.3-1.0mm thick. From these tension experiments it was determined that an Alginate:PEG ratio of [4:1] (Figure 5-6) yielded the highest average modulus (~20,000kPa).

CaCl Concentration: Based on the results above, CaCl concentrations ([0.5M], [1.0M], [2.0M])(Figure 5-6) were varied using gels composed of an Alginate:PEG ratio of "[4:1]". Statistical comparisons were made between the experimental results and a commercial silicone tube control. Alginate:PEG ratios of [4:1] along with the [0.5M] and [1.0M] CaCl crosslinking solutions were all significantly stronger then the commercially available silicone tubes in tension with [0.5M] CaCl achieving a maximum value (~50,000kPa) (*P-value < 0.05).

Drying Time: Gels composed of an Alginate:PEG ratio of [4:1] crosslinked with [0.5M] CaCl yielded the strongest overall modulus (from above) and were used to determine the optimal gel drying time. Results show drying times greater then 4 days produce gels that are significantly stronger than commercial silicone tube controls in tension (*P-value <0.05) (Figure 5-7). Mechanical strength levels off after 4 days with minimal increases up to 30 days. Based on these results, the strongest gel combinations were achieved using an Alginate:PEG ratio of [4:1] crosslinked with a CaCl concentration of [0.5M] that had been dried for at least 4 days.

Maximize Mechanical Strength (Compression)

Compression Testing: Alginate gel tube compressive strength was determined by measuring the force required to compress the gel tubes 25%. The results follow a similar trend to those from the tension experiments (Figure 5-8, 5-9) where the strongest gel tube in compression was composed of an Alginate:PEG ratio of [4:1] crosslinked with a CaCl concentration of [0.5M] (Figure 5-8) and allowed to dry for at least 1 day (Figure 5-9). All ingredient concentrations and combinations were significantly stronger than the commercially available silicone tube in compression (Figure 5-8) (*P-value < 0.05). Furthermore, in compression, gel samples dried for longer then 1 day (Figure 5-9) were all significantly stronger then the commercially available silicone tube (*P-value < 0.05).

Physiological Experiments

After determining the mechanical properties of the alginate gels in tension and compression physiological experiments were conducted.

Quantify compressive strength after agar encapsulation

In-vivo tissue encapsulation was simulated by encapsulation within an in-vitro agar substrate. Mechanical (compressive) strength of alginate gel tubes ([4:1] Alginate:PEG crosslinked with [0.5M] CaCl and dried for 7 days), after encapsulation within an agar substrate (Pluronic F-127), was measured over 15 and 30 days. Results show a maximum of 50% loss (Day 15) in mechanical compressive strength over the 30 days compared with a non-encapsulated control (Figure 5-10). Fifteen day encapsulation results were significantly weaker than the alginate control (*P-Value = 0.014). However, 30 day results were statistically similar to those of the control (*P-Value = 0.299). Fifteen day results are not significantly lower than (Day 30) results (*P-Value=0.087). However, all tubes encapsulated within the agar were significantly stronger than the silicone tube in compression (*P-Value < 0.05)

Quantify compressive strength from exposure to ototopical solutions

Children are the primary benefactors of TT implantation and occasional contact with ototopical solutions can occur. Alginate gel compressive strength after 24 hour exposure to these ototopical solutions was determined. Tubes were composed of Alginate:PEG [4:1] crosslinked with [0.5M] CaCl, and dried for 7 days. Ototopical solutions included pool water, soap water, salt water, H_2O_2, Blood Serum, Vinegar, Ear Mucus, Ciprodex, and Ofloxacin. Results show that exposure to Ofloxacin (antibiotic) yields the largest drop-off in compressive strength of (73%). Compressive strength was conserved in alginate tubes exposed to blood serum (47% decrease from control) (Figure 5-11). Ear Mucus showed a decrease in compressive strength of 58%. All ototopical treatments reduce the compressive strength significantly (*P-Value < 0.05),

from the no treatment alginate control. However, none are below the compressive strength of the commercial silicone tube which is more than 90% lower than the alginate control and greater then 60% lower than the most detrimental treatment (P-value < 0.05).

Minimize alginate gel tube plugging

The plugging rate of the alginate reuter-bobbin tube was measured using an in-vitro ear chamber. Ten commercially available stainless steel reuter-bobbin tubes and 20 Alginate:PEG [4:1], CaCl [0.5M], 7 day dried gel tubes (10 uncoated and 10 coated with human serum albumin (HSA)) were tested in the chamber. Occlusion results were compared to a previously published report showing the in-vivo plugging rate of a stainless steel reuter-bobbin tube to be 74% [52]. The experimental outcome showed uncoated alginate gel tubes with a plugging rate of 50%, while the HSA coated alginate tubes had a plugging rate of 30%. Results indicate that the process of submerging/coating alginate tubes in HSA limits the formation of an occlusion. Glass rods were re-inserted after HSA application to reform the tube lumen due to swelling resulting from re-hydration. Uncoated stainless steel reuter-bobbin tubes had a plugging rate of 70% using the in-vitro ear chamber (Figure 5-12). HSA coated alginate tubes were 50% less likely to occlude than the stainless steel tubes (30% plugging vs. 70% plugging) while un-coated alginate tubes were 20% less likely to occlude. Survival distribution function curves (Kaplin-Meier Method) show that the HSA coated tubes resist occlusion over a significantly longer time period than commercial stainless steel tubes (*P-value = 0.047) (Figure 5-13).

Specific Aim 2: Optimize the Dissolution Components for Complete Dissolution

Dissolution Solutions

Removal of alginate gel TTs from the tympanic membrane can be achieved with the use of a dissolution solution. The rate of dissolution was measured after exposure to several treatments (NaCl, Vinegar, $NaHCO_3$). Gel tubes, of various compositions, were exposed (complete

submersion) to each treatment for several hours (1-5 hours) and given an R-value based on the amount of dissolution that had occurred. Results were averaged and graphed (Figure 5-14). Analysis shows that while dissolution is maximized (R-value = 4.5) in gels composed of an Alginate:PEG ratio of [1:2], mechanical strength is limited (1 hour). Tubes composed of an Alginate:PEG ratio of [4:1] (high mechanical strength) achieved an R-value of 3.5 after only one hour, using a 50/50 mixture of NaCl and $NaHCO_3$ [1.0M] and $NaHCO_3$ alone [1.0M] (Figure 5-14). Dissolution continued to increase, while solution was present, from 1 to 5 hours (Figure 5-15). Vinegar and vinegar+$NaHCO_3$ proved to be minimally effective in removing alginate tubes. Although promising, NaCl+$NaHCO_3$ did not perform (dissolve after 1 hour) as well as the $NaHCO_3$ alone across all samples. On the other hand, sodium bicarbonate's ($NaHCO_3$) dissolution performance as well as its use in previous clinical studies provides a viable method for removing TTs after insertion. Therefore, sodium bicarbonate was used as the dissolution solution for the remaining experiments.

Dissolution Concentration

The quantity of $NaHCO_3$ present in solution may alter the rate of tube dissolution. As a result, $NaHCO_3$ concentration was increased from [0.1M], to [0.5M], [1.0M], and [2.0M] while measuring dissolution (R-value). Results show that dissolution is not linearly related to $NaHCO_3$ concentration, rather dissolution peaks at [0.5M] (R=5, 5 hours) followed by decreased dissolution (Figure 5-16). Over time (1-5 hours), dissolution increases significantly (P-Value $<$ 0.05) across all treatments.

Physiological Experiments

After determining the optimal dissolution concentration and ingredients, remaining dissolution experiments were performed within an in-vitro ear chamber which limited dissolution access to one side of the tube.

Effect of desiccation on dissolution

Throughout implantation (drying in an incubator at 37°C) moisture was removed from the tube. The effect of increased drying time (0, 15, 30 days at 37°C) on dissolution was measured within an in-vitro ear chamber (See Figure 5-17). The dissolution conditions within the in-vitro ear chamber were similar to those encountered during in-vivo dissolution. Results show that the alginate tubes dissolve in two steps: first the barrel portion dissolves away from the flange, followed by swelling and breaking apart of the flange portion. After one hour, the flange portion of the each of the tubes had only partially dissolved and incomplete dissolution was seen in the majority of the tube barrels. Then, additional dissolution solution was applied to the tubes to replace solution lost to evaporation. After two hours the fresh tubes (flange and barrel) had dissolved to R=4.2 while the 15 and 30 day tubes (flange and barrel) had dissolved to an R=5.3 and R=5.5 respectively (Figure 5-17). All dried tubes dissolved within two hours based on the R value. Analysis shows that fresh tubes or tubes with a higher percentage of internal moisture dissolve at a slower rate then those dried for extended periods of time (*P-Value < 0.05).

Dissolution of HSA coated alginate tubes

Previous experiments have shown that HSA coated tubes limit tube plugging (Figure 5-12) and increase performance. However, it was not known what effect HSA coatings have on dissolution. As a result, tubes coated in HSA solution were dissolved in an in-vitro ear chamber using [0.5M] $NaHCO_3$ for 1-5 hours and compared to un-coated alginate tubes. Results show that HSA coatings do not negatively affect dissolution (Figure 5-18). After 5 hours all tubes coated in albumin were completely dissolved (R=5.5). No significant difference was measured between HSA coated and un-coated alginate tubes when dissolved in [0.5M] $NaHCO_3$ (*P-Value > 0.05). Dissolution results are summarized in Figure 5-19 and Table 5-1.

Specific Aim 3: Quantify Ototoxicity from Exposure to Dissolution Products

Pilot Study Results

Dissolution solution concentration and treatment exposure times were optimized based on morbidity changes. $NaHCO_3$ + (dissolution solution along with dissolved alginate gel tubes) concentration was reduced from 0.5M to 0.1M while reducing the exposure time (24 hours, 1 hour, 30 minutes, 15 minutes, 10 minutes, 5 minutes). Cisplatin concentration was simultaneously increased to obtain measurable results within short exposure times. Survival and ototoxicity results based on treatment and exposure time was recorded (Table 5-2). Results show that the highest concentration tolerated by the Zebrafish was 0.1M $NaHCO_3$ + for a duration of 30 minutes. The Cisplatin concentration necessary to elicit a measurable result after 30 minutes was 1mM.

Ototoxicity after Treatment Exposure

After optimization, ototoxicity experiments based on exposure to the above treatments (dissolution products and controls) were conducted. Zebrafish hair cells were isolated and analyzed via fluorescence under a UV microscope (Figure 5-20). Fluorescent intensity, neuromast area, and hair cell quantity were measured and compared, at neuromast (P4) between all three treatments (dissolved and filtered alginate gel tubes, Cisplatin solution, and embryo media solution). Figure 5-21 and Figure 5-22 show the mean fluorescent intensity between all three treatments (after normalization to the no-treatment control) when compared to the negative (No-Treatment) embryo media control. Analysis shows that Zebrafish hair cells exposed to dissolution products have a significantly higher fluorescent intensity (*P-value = 0.032) than those exposed to the positive Cisplatin control. Furthermore, there was no difference in fluorescent intensity (*P-value = 0.322) between the negative (No-Treatment) control and the dissolution products proving that the dissolution products have little to no effect on hair cell

mortality under these specific conditions. There is however, a measurable difference in fluorescent intensity between Zebrafish hair cells exposed to both the negative and positive control (*P-value = 0.004).

Moreover, neuromast size (area of fluoresced hair cells) is directly related to the number of viable hair cells present within each neuromast. Figure 5-23 shows the relationship between neuromast size after a 30 minute treatment exposure. Results show that Zebrafish hair cells exposed to dissolution products have a significantly larger neuromast size than those exposed to the positive Cisplatin control (*P-Value=0.004). The results were similar to the fluorescent intensity results with no differences in neuromast size between the negative (No-Treatment) control and the dissolution solution (*P-Value=0.235) and significant differences in neuromast size between both the negative and positive control (*P-Value=0.000).

Furthermore, the number of viable hair cells located within neuromast P4 varied depending on the exposure treatment. Results show that hair cell quantity is directly related to treatment. Figure 5-24 shows that both the negative (No-Treatment) control and the dissolution products have significantly greater numbers of viable hair cells than the positive (Cisplatin) control (*P-Value=0.039 and *P-Value=0.000 respectively). Results are summarized in Appendix D.

Additionally, dissolution experiments were re-done using the lower dissolution concentration (0.1M $NaHCO_3$) and exposure time (30 minutes) to determine if dissolution can be achieved. Within the 30 minute exposure time no changes were seen in dissolution of the alginate gel. However, results show that tubes dissolved within 0.1M $NaHCO_3$ required ~24 hours to completely dissolve (R=5.5).

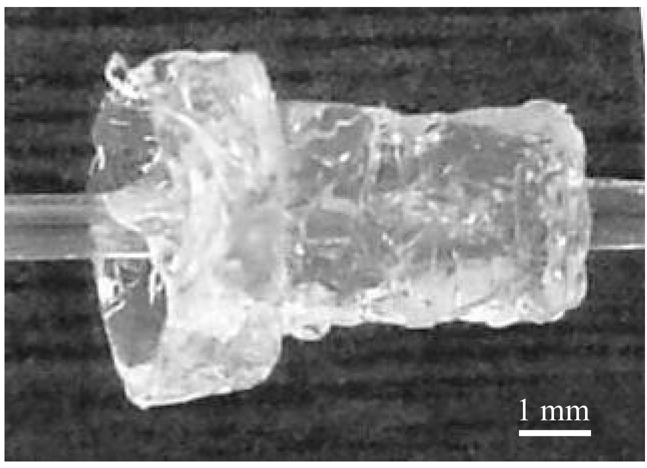


Figure 5-1: Fresh Calcium alginate/PEG T-tube on glass curing rod.

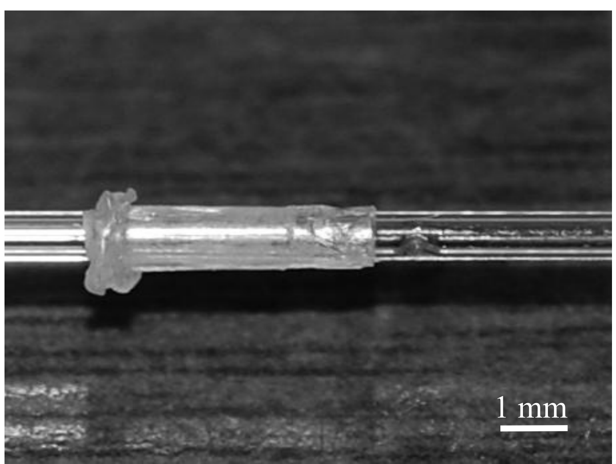


Figure 5-2: Dried Calcium alginate/PEG T-tube on glass curing rod.

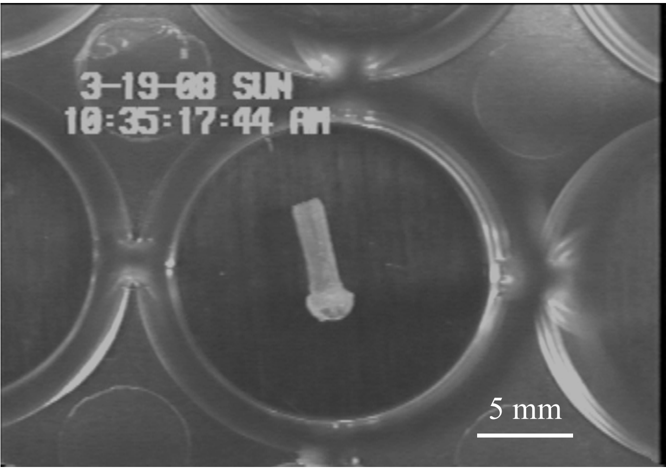


Figure 5-3: Dried Calcium alginate/PEG T-tube removed from glass curing rod and placed in dissolution well.

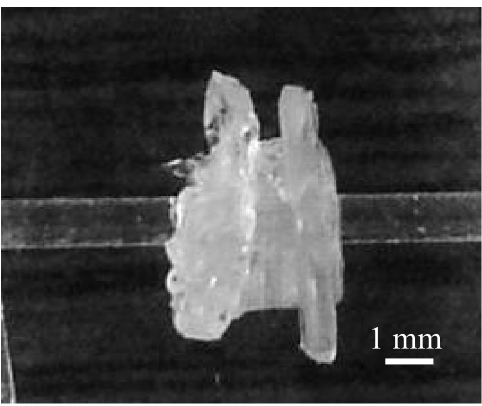


Figure 5-4: Fresh Reuter Bobbin Tube. Note: one flange (right side) is completely in tact while left flange is missing part due to the manufacturing.

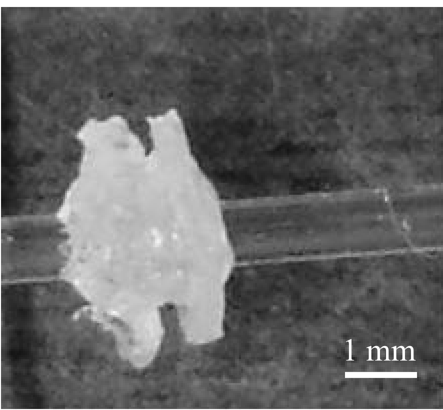


Figure 5-5: Dried Reuter Bobbin Tube. Note: tube overall diameter has decreased due to shrinkage during drying.

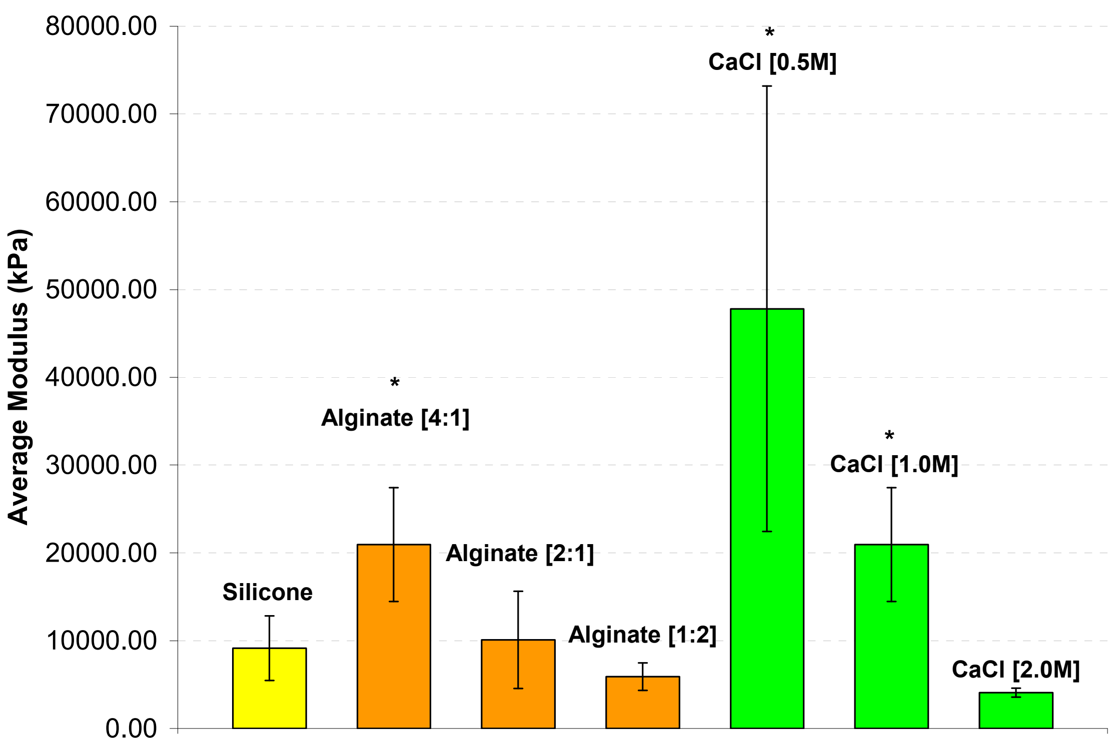


Figure 5-6: Mechanical testing (Tension) average modulus results for Alginate:PEG ratio and CaCl concentrations when compared to commercially available silicone tubes. The calcium concentrations were made using an Alginate:PEG ratio of [4:1]. (* indicates mechanical strength is significantly greater then silicone control, P-value <0.05) (n=10)

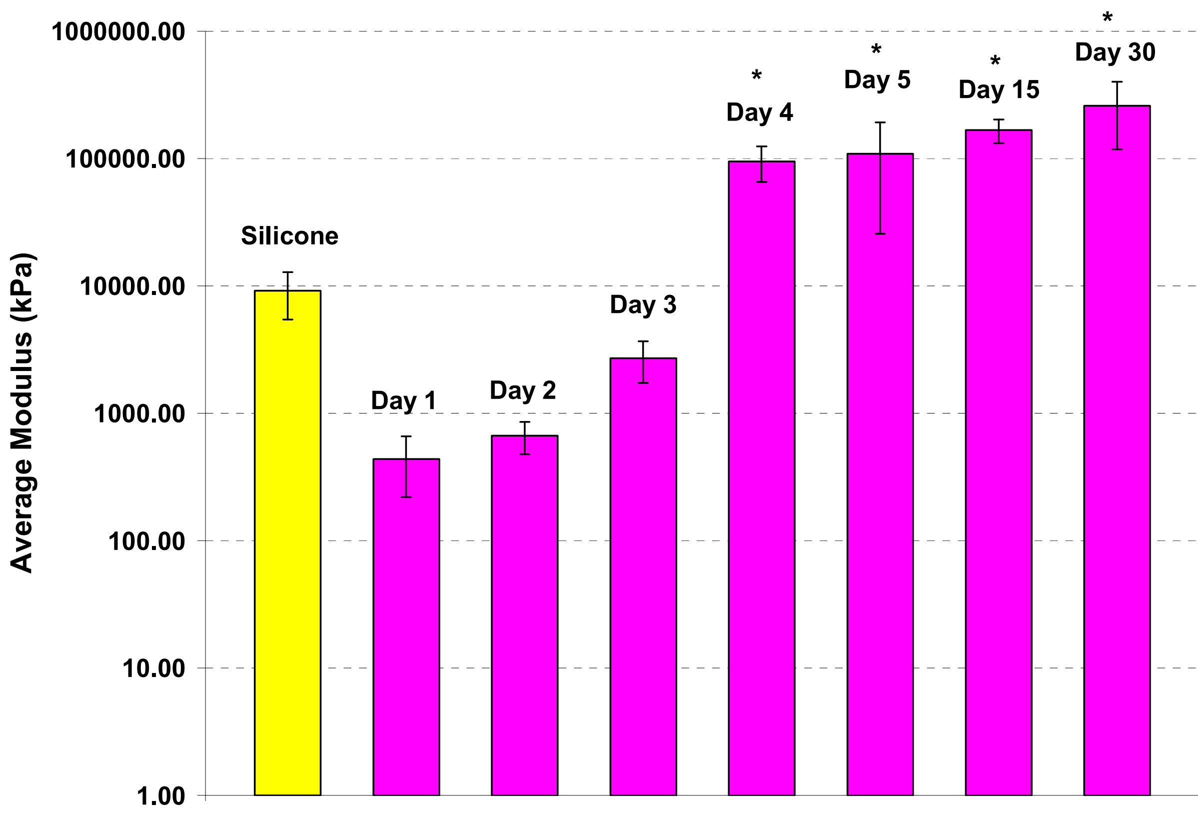


Figure 5-7: Mechanical testing (Tension) results for the effect of desiccation time on the mechanical strength of the alginate gel when compared to commercially available silicone. (* indicates mechanical strength is significantly greater then silicone control, P-value <0.05) (n=10)

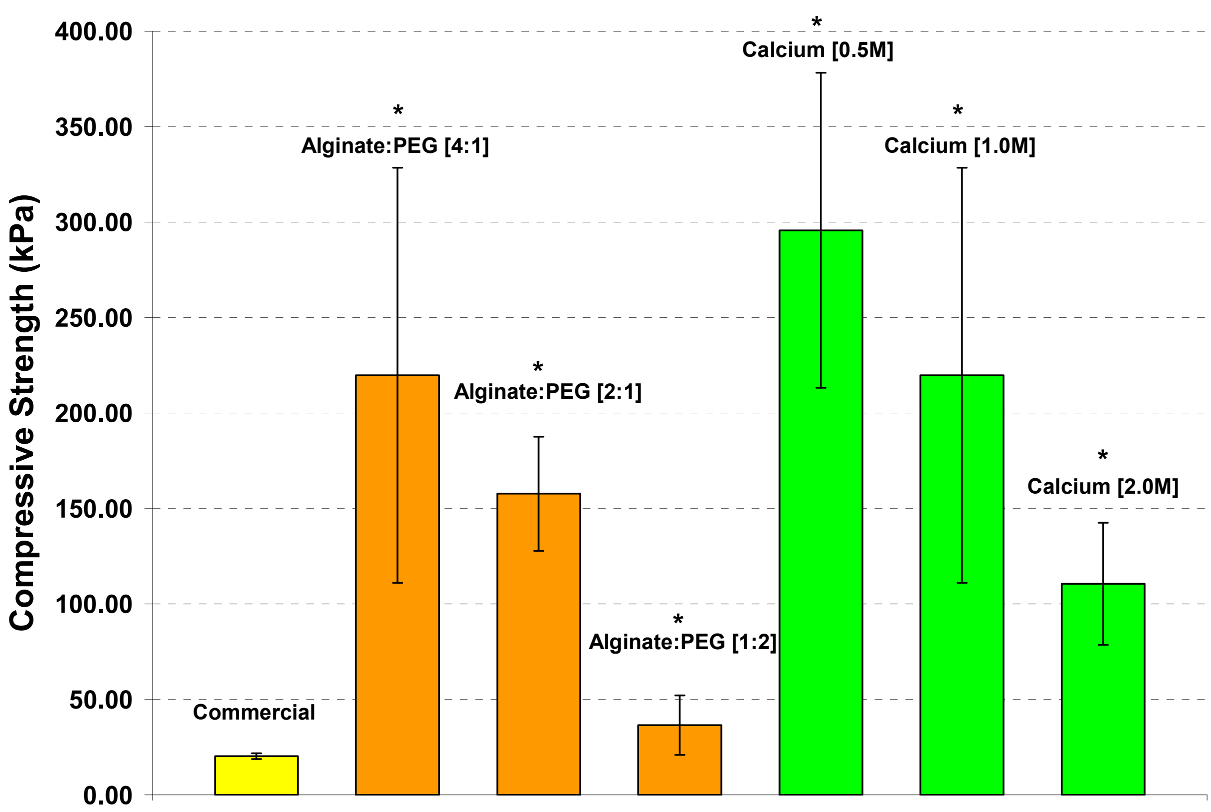


Figure 5-8: Compressive strength of the Alginate:PEG gels based on alginate composition and CaCl crosslinking concentration. All results are compared to a commercially available silicone Goode tube. The calcium concentrations are made using an Alginate:PEG ratio of [4:1]. (* indicates mechanical strength is significantly greater then silicone control, P-value <0.05) (n=10)

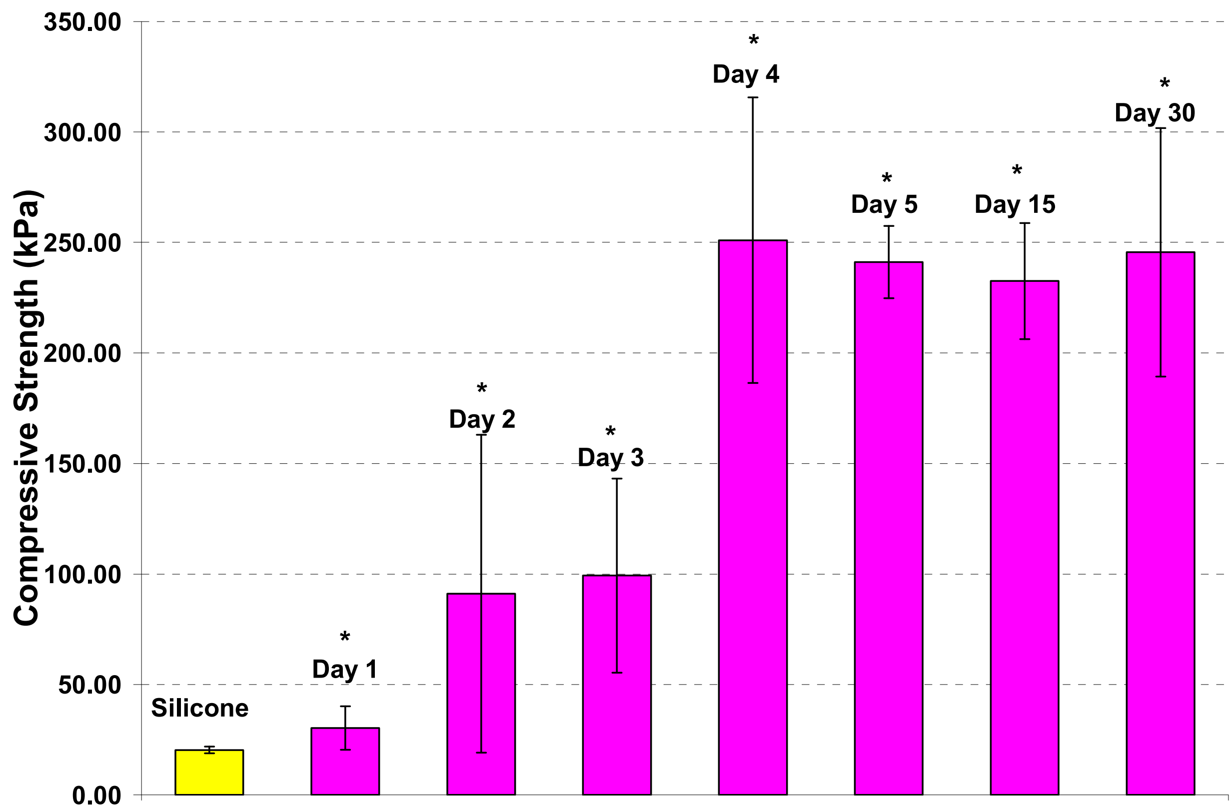


Figure 5-9: Compressive strength of the Alginate:PEG gels based on desiccation time. All results are compared to a commercially available silicone Goode tube. (* indicates mechanical strength is significantly greater then silicone control, P-value <0.05) (n=10)

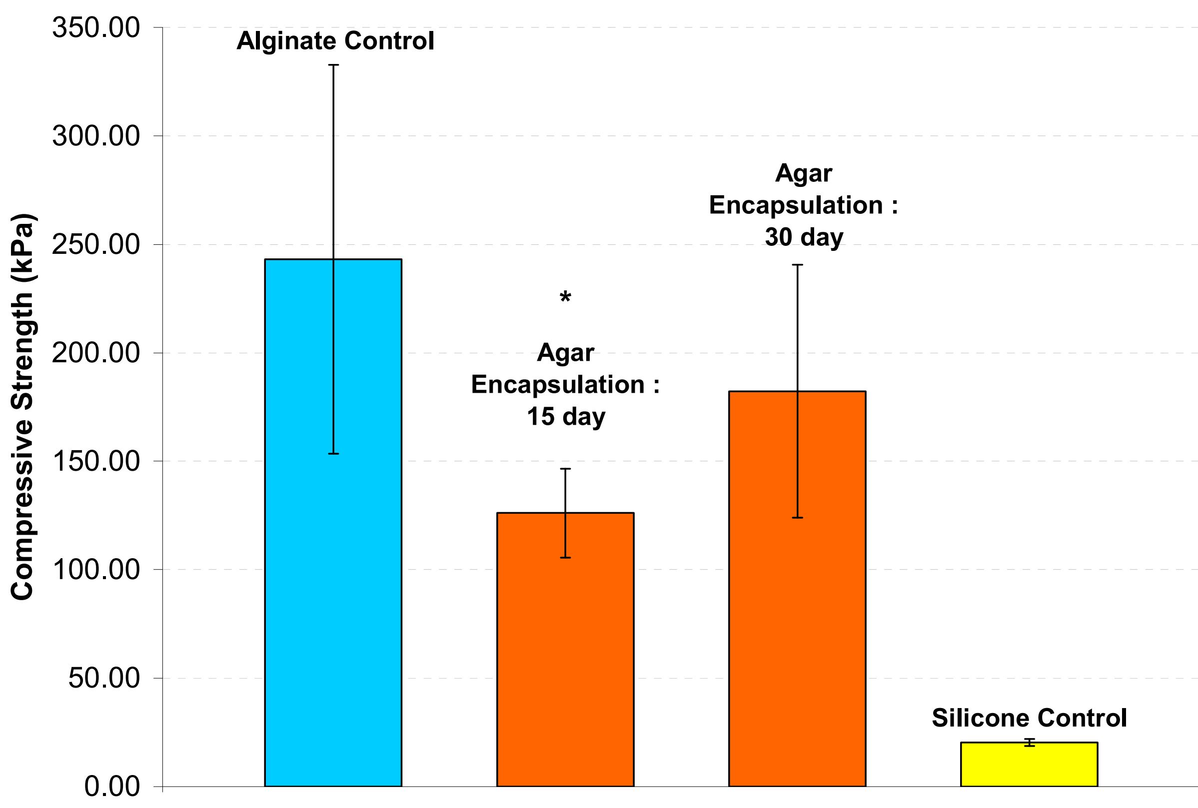


Figure 5-10: Compressive strength of agar encapsulated alginate gel tubes after 15 and 30 days compared to non-encapsulated alginate gel tube and silicone control. (n=5)

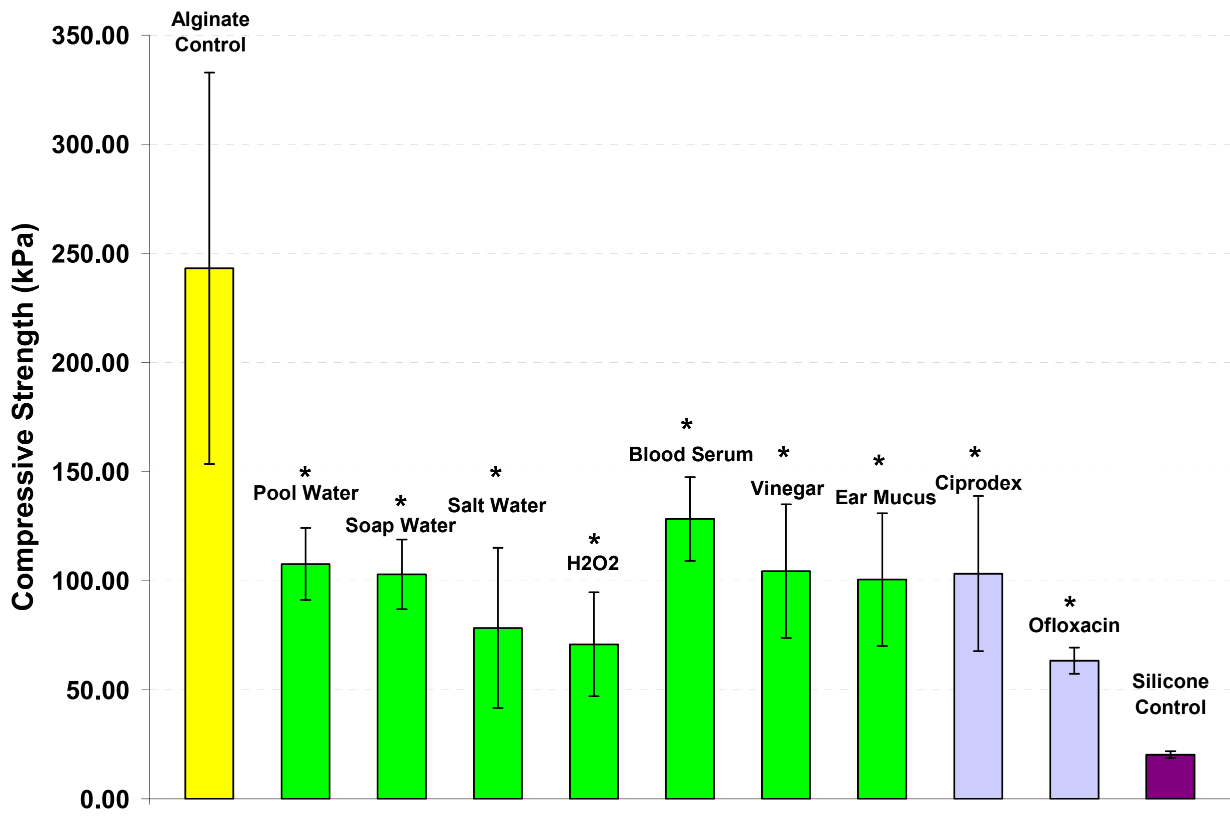


Figure 5-11: Compressive strength of alginate gels exposed to various ototopical solutions for 24 hours. Most detrimental treatment was Ofloxacin (73% decrease) while least detrimental was blood serum (47% decrease). All treatments are significantly stronger then the silicone control sample and significantly lower then the alginate control sample (P-Value <0.05). (n=5)

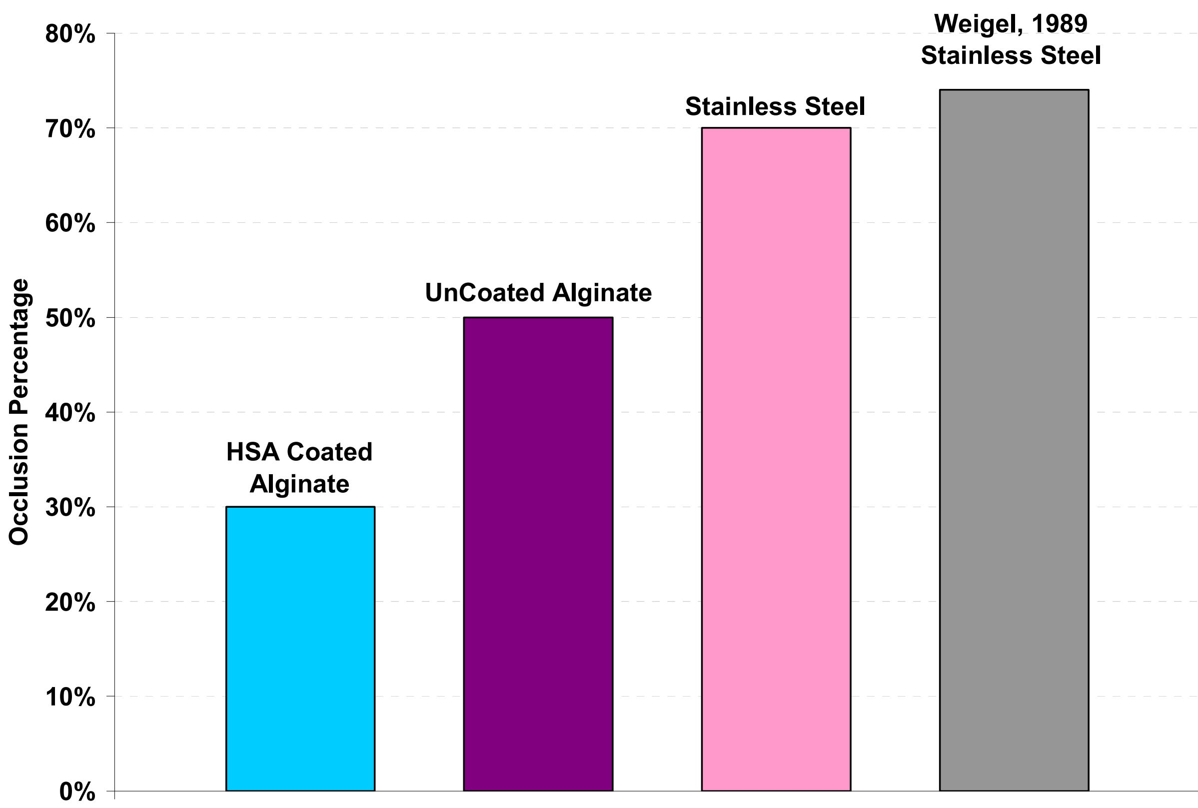


Figure 5-12: Occlusion percentage of the alginate gel tubes (HSA coated and uncoated) compared to the uncoated stainless steel tubes using the in-vitro ear chamber. Results compared to published in-vivo stainless steel occlusion rates from Weigel et al. 1989 (n=10).

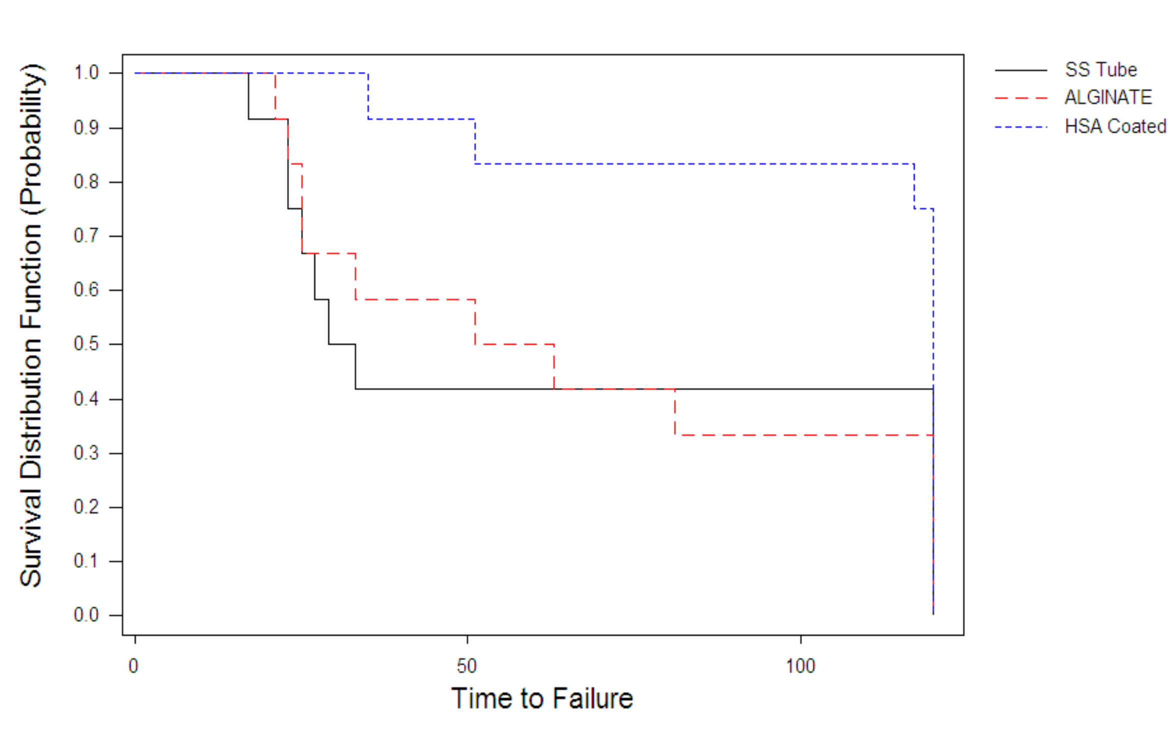


Figure 5-13: Nonparametric Survival Plot (Kaplin-Meier Method) for stainless steel, uncoated alginate, and HSA coated alginate tubes. Tubes were tested in the in-vitro ear chamber. (n=10)

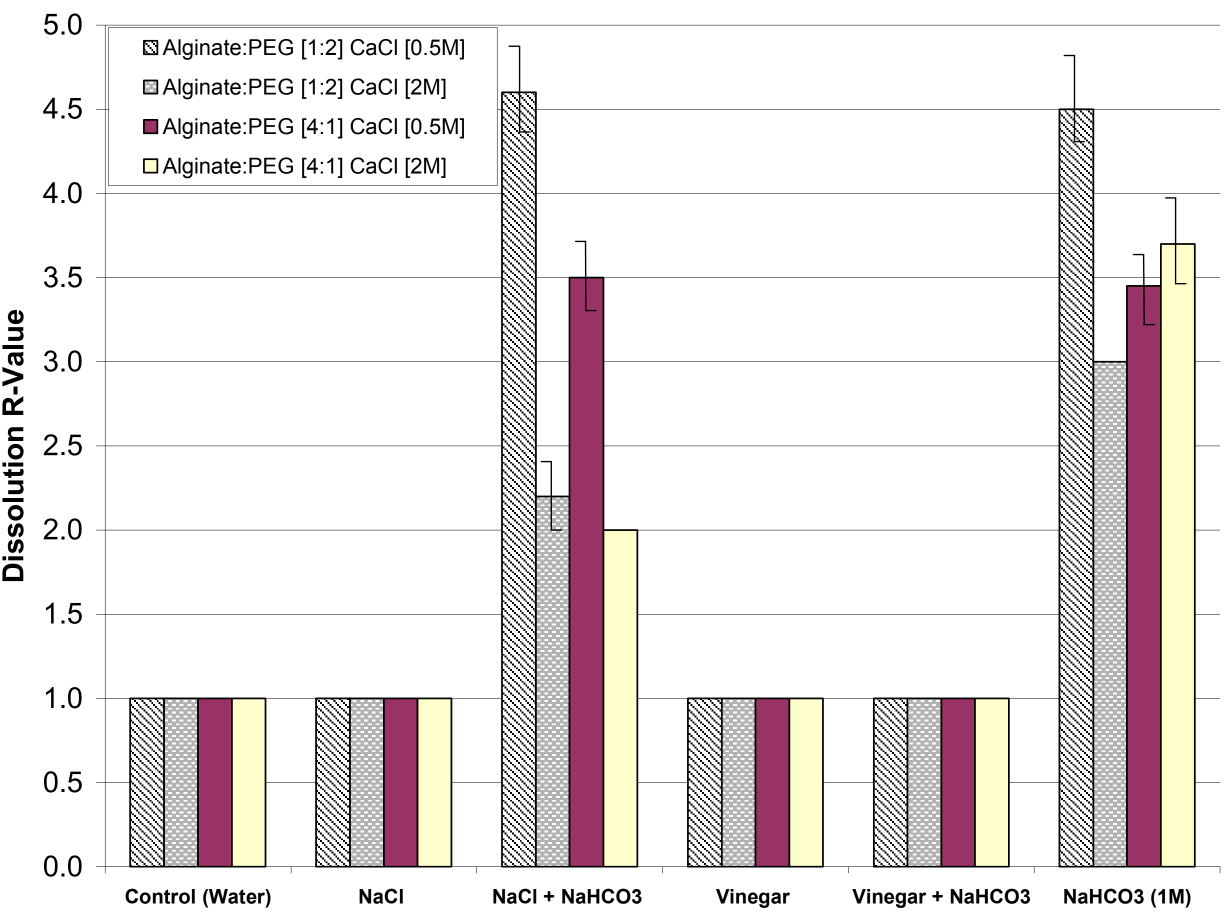


Figure 5-14: Dissolution results for additives (NaCl and Vinegar vs. $NaHCO_3$) over 1 hour. Tubes were completely submerged within the dissolution solution. (n=5)

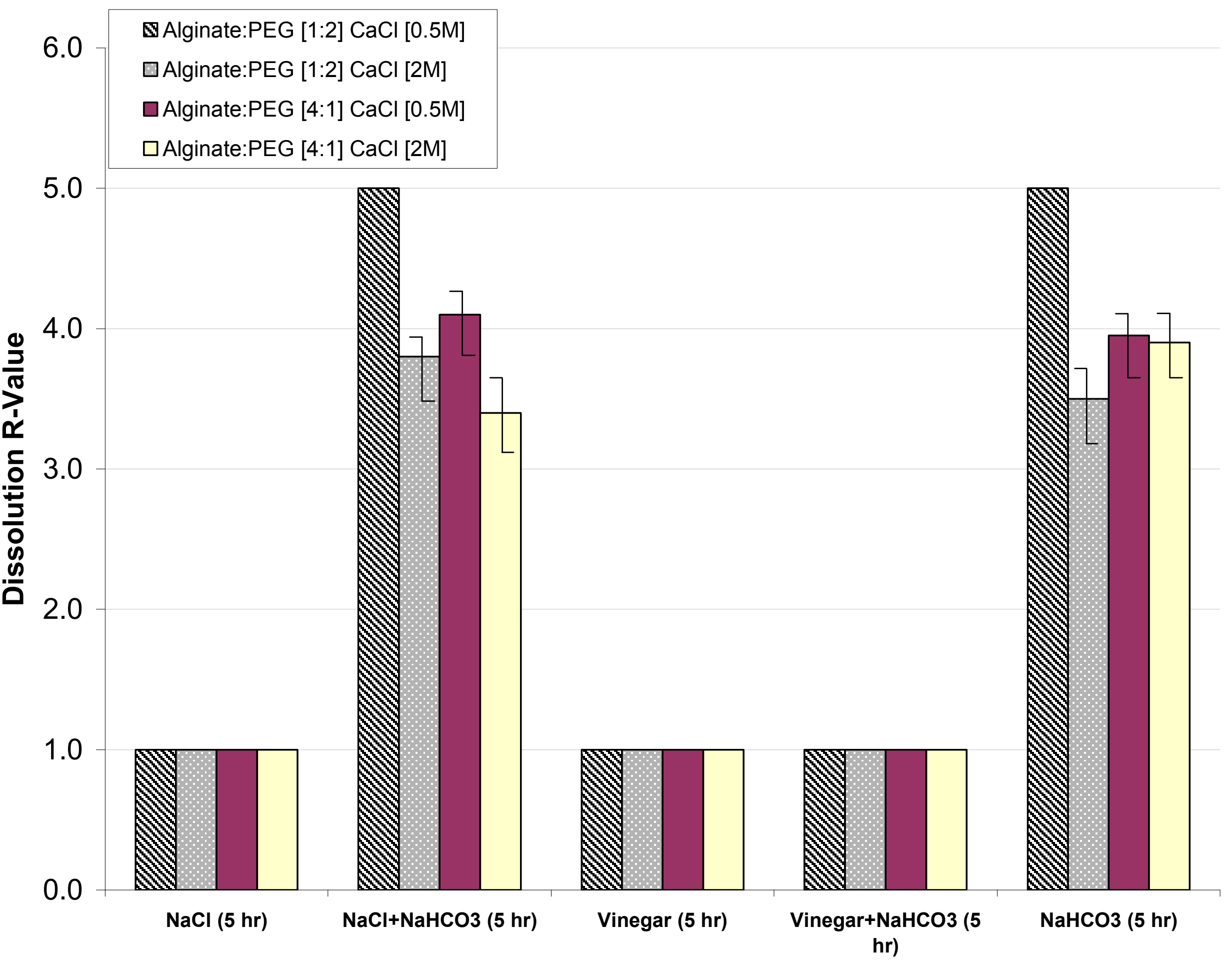


Figure 5-15: Five hour dissolution results for additives (NaCl and Vinegar vs. $NaHCO_3$). Shows how dissolution continues in the presence of the dissolution solution. (n=5)

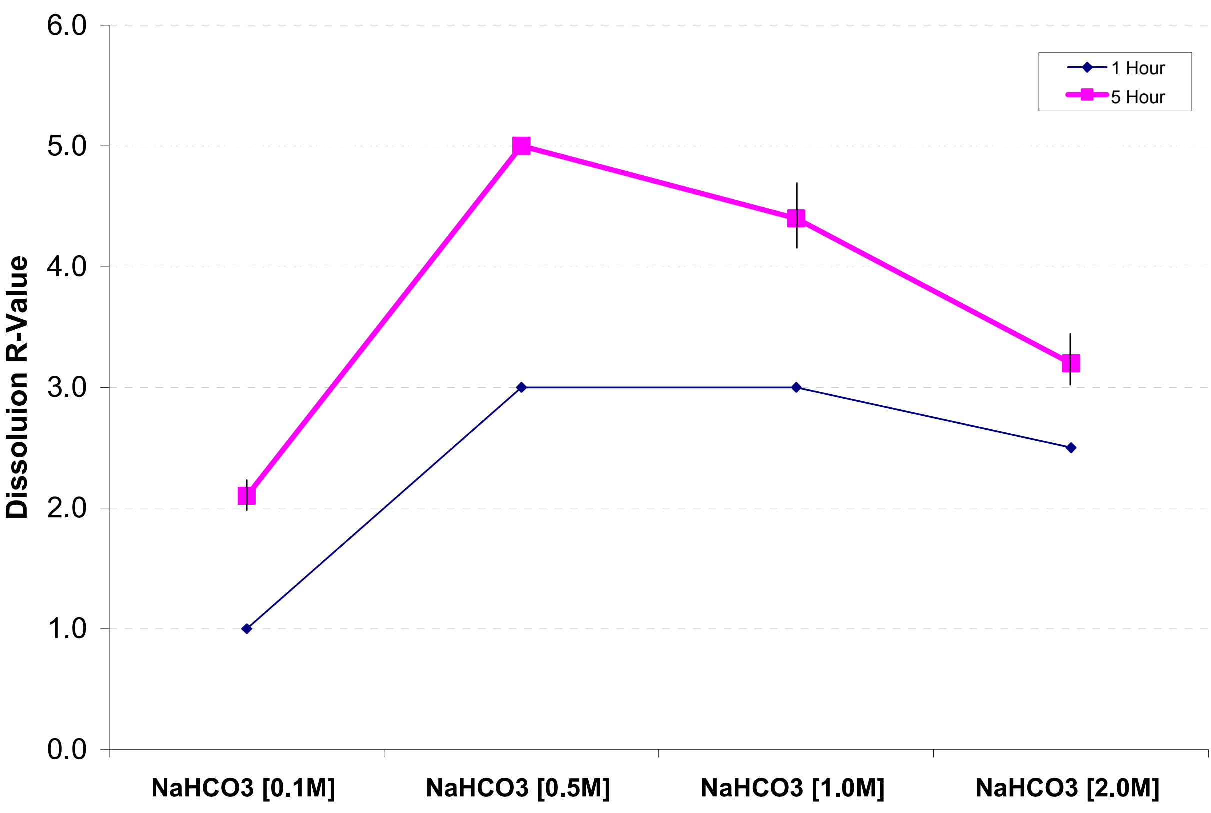


Figure 5-16: Dissolution results based on $NaHCO_3$ concentration (0.1M, 0.5M, 1.0M, 2.0M) over 1-5 hours. All 5 hour results are dissolved significantly more then 1 hour results (P-Value < 0.05). Tubes were completely submerged within the dissolution solution. (n=5)

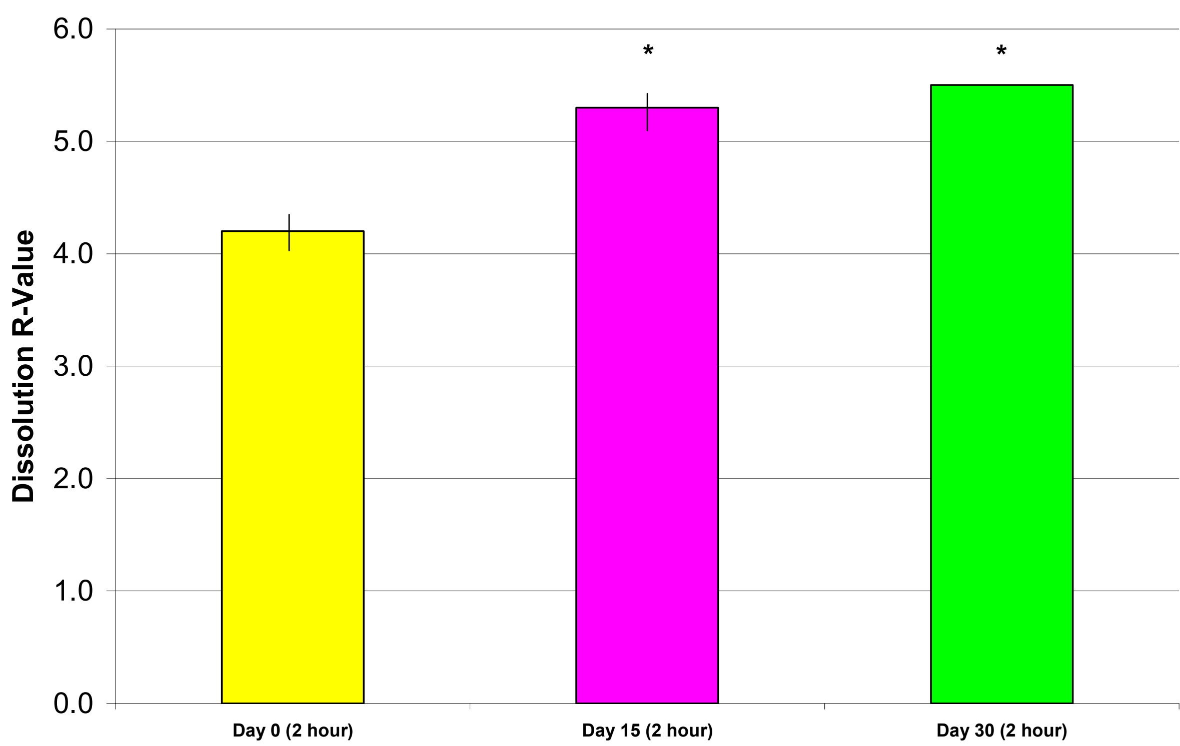


Figure 5-17: Effect of drying time (0, 15, 30 days dried) on the dissolution rate of the gel tube. 15 and 30 Day drying times dissolved significantly faster then fresh (Day 0) tubes. (* P-Value < 0.05). Tubes were dissolved within an in-vitro ear chamber. (n=5)

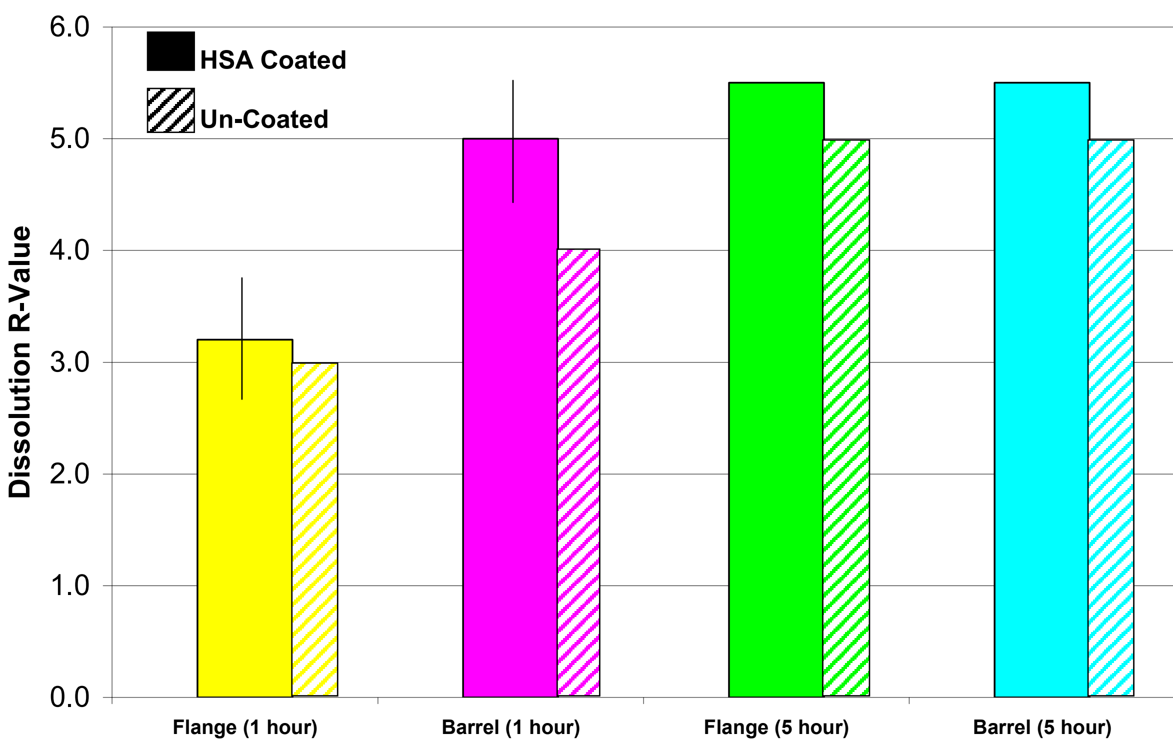


Figure 5-18: Effect of HSA coating on the dissolution rate of the alginate gel tubes. Comparison between flange and barrel portions as well as between un-coated/coated alginate tubes dissolved in [0.5M] $NaHCO_3$. (No significant difference was seen between HSA Coated and Un-Coated tubes (P-Value > 0.05)) (n=5)

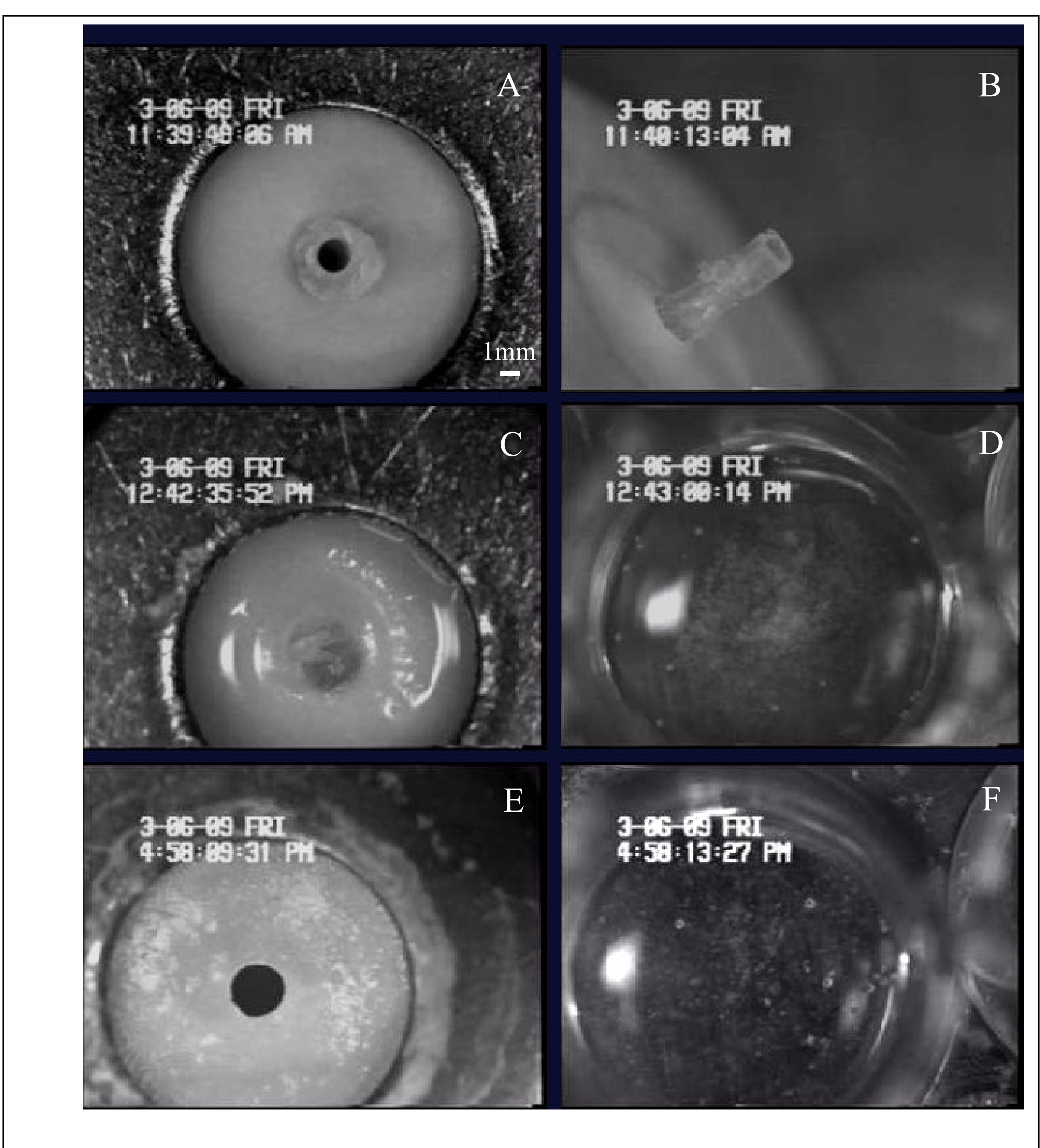


Figure 5-19: Dissolution within ear chamber over time (1-5 hours). (A) Front of TT (facing flange), showing depression for dissolution solution (t=0). (B) Back of TT (facing barrel), showing latex conforming around barrel of tube (t=0). (C) Front of TT (flange), showing dissolution solution and deformed TT flange (t=1hr). (D) Well "middle ear space" with excess dissolution solution, barrel dissolution (t=1hr). (E) Front of TT (flange), completely dissolved tube and hole in elastic membrane (t=5hr). (F) Well "middle ear space" where dissolution of barrel occurs (t=5hr).

Table 5-1: Dissolution Results Based on Treatment and Dissolution Time

Treatment	1 Hour Dissolution	2 Hour Dissolution	5 Hour Dissolution	24 Hour Dissolution
NaCl	None	None	None	None
NaCl + $NaHCO_3$ (1M)	Partial/Complete	Partial/Complete	Partial/Complete	--
Vinegar	None	None	None	--
Vinegar + $NaHCO_3$ (1M)	None	None	None	--
$NaHCO_3$ (0.1M)	None	None	None	Complete
$NaHCO_3$ (0.5M)	Partial	Partial	Complete	--
$NaHCO_3$ (1.0M)	Partial/Complete	Partial/Complete	Partial/Complete	--
$NaHCO_3$ (2.0M)	Partial	Partial	Partial	--
0 Day Drying	Partial	Partial	--	--
15 Day Drying	Partial	Complete	--	--
30 Day Drying	Partial	Complete		
HSA Coated (flange)	Partial	Partial	Complete	--
HSA Coated (barrel)	Complete	Complete	Complete	--

None = R-Value < 2; Partial = R-Value < 5; Complete = R-Value ≥ 5

Table 5-2: Survival and Ototoxicity Results Based on Treatment and Exposure Time

	24 hours	1 hour	30 minutes	15 minutes	10 minutes	5 minutes
0.5M $NaHCO_3$ +	X	X	X	X	X	X
0.25M $NaHCO_3$ +	—	—	X	X	X	X
0.1M $NaHCO_3$ +	X	X	SURVIVAL	Survival	Survival	Survival
50µM Cisplatin	Measurable Ototoxicity	X	X	X	X	X
1mM Cisplatin	—	—	MEASURABLE OTOTOXICITY	Measurable Ototoxicity	—	—

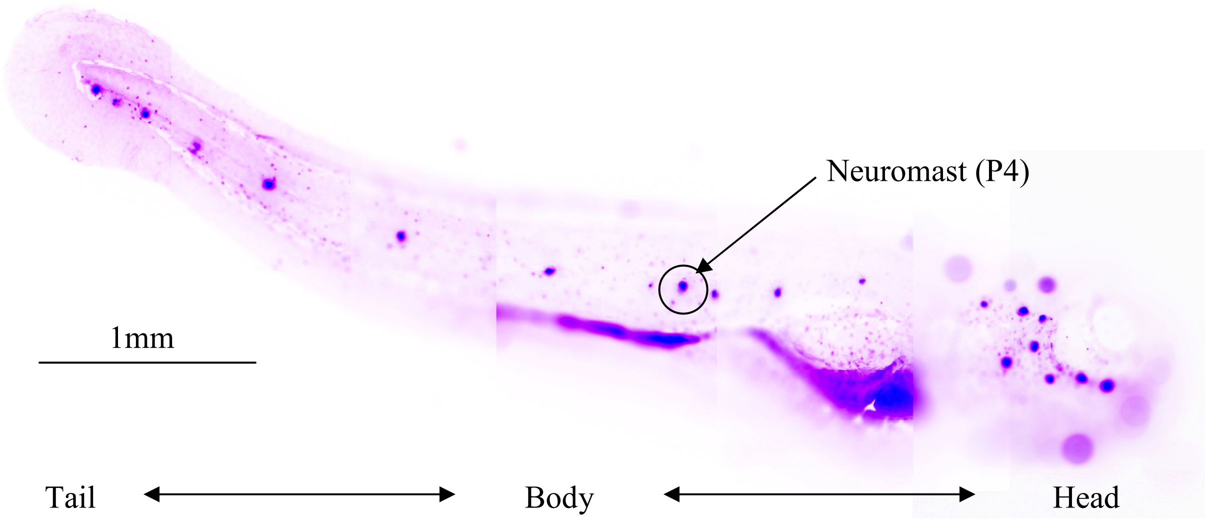


Figure 5-20: Whole fish fluorescence after DASPEI stain. Neuromasts located along the head and length of the body. Stomach and gut readily absorb DASPEI stain shown as dark purple regions. Highlighted region represents neuromast P4.

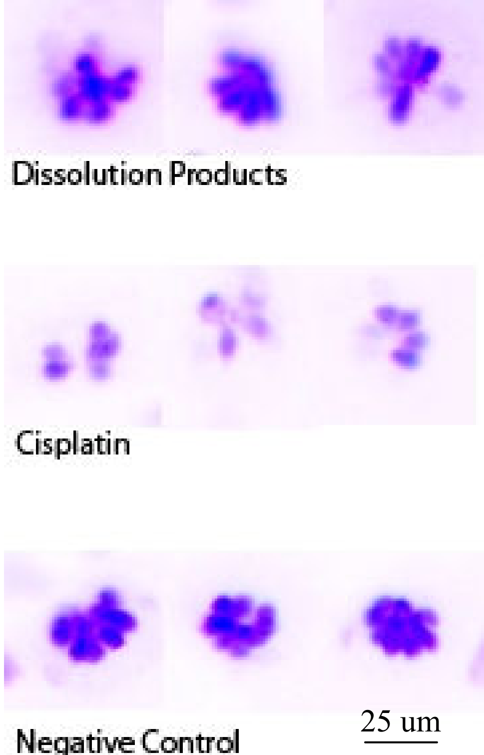


Figure 5-21: Fluorescent intensity for all three treatments (Dissolution Products, Positive Cisplatin Control, Negative (No-Treatment) Control). Both dissolution products and negative (No-Treatment) control neuromasts have very high fluorescent intensity (dark purple) whereas Cisplatin (a known carcinogen) have very low fluorescence (light purple). (n=18)

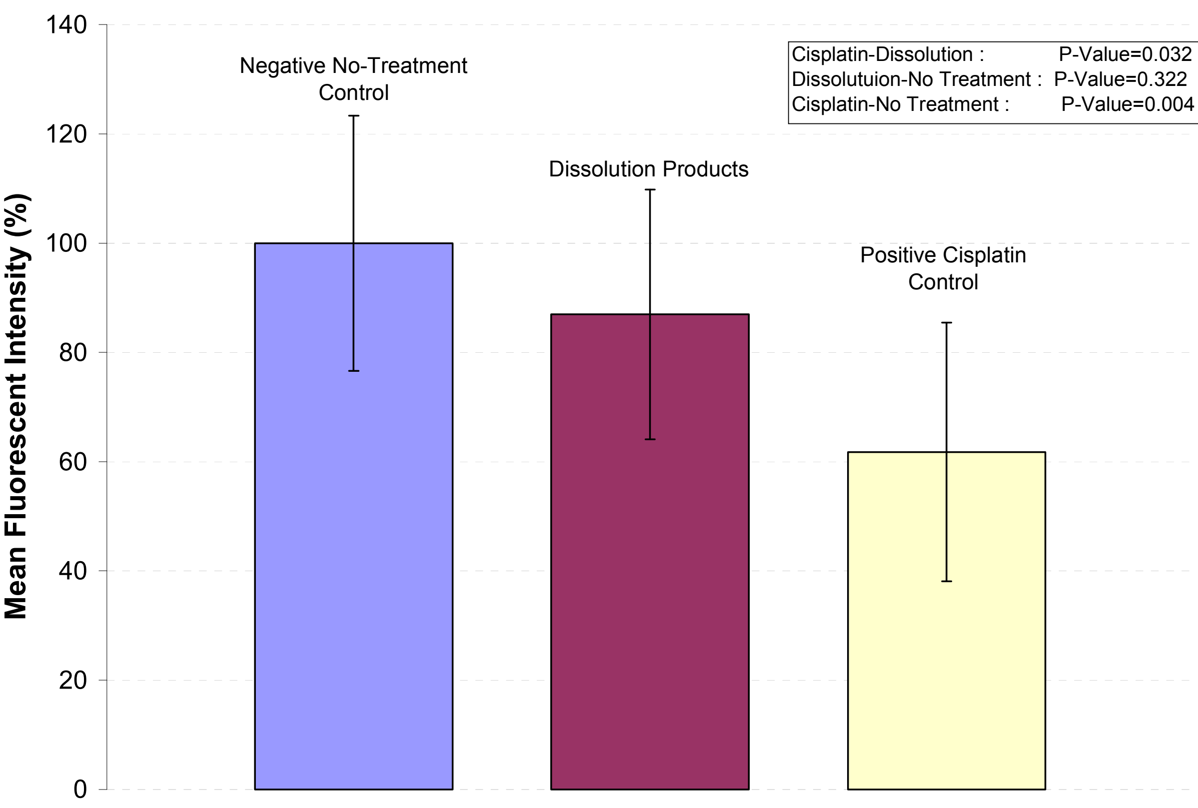


Figure 5-22: Mean fluorescent intensity between the positive, negative, and dissolution product treatments. Results normalized to the negative (No-Treatment) control. (n=18)

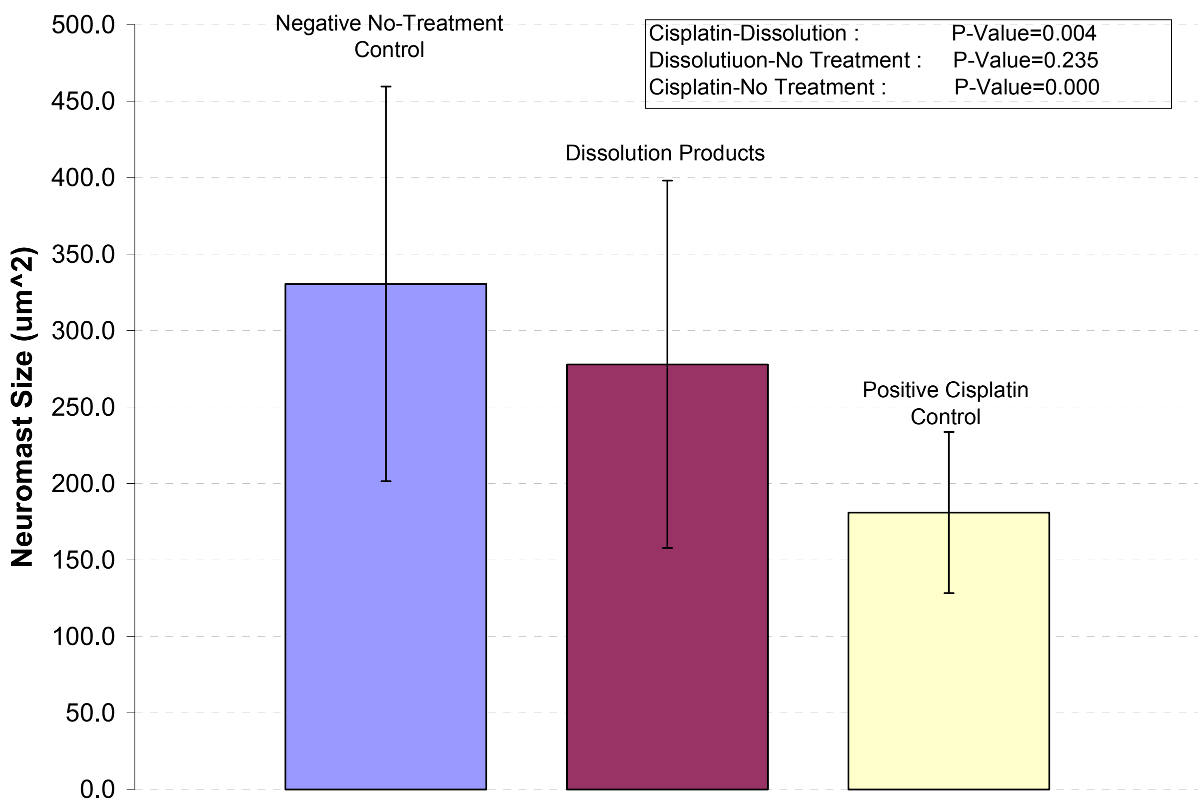


Figure 5-23: Neuromast size based on exposure treatments. Both the negative (No-Treatment) control and the dissolution products maintained a higher neuromast size then the positive Cisplatin control. (n=18)

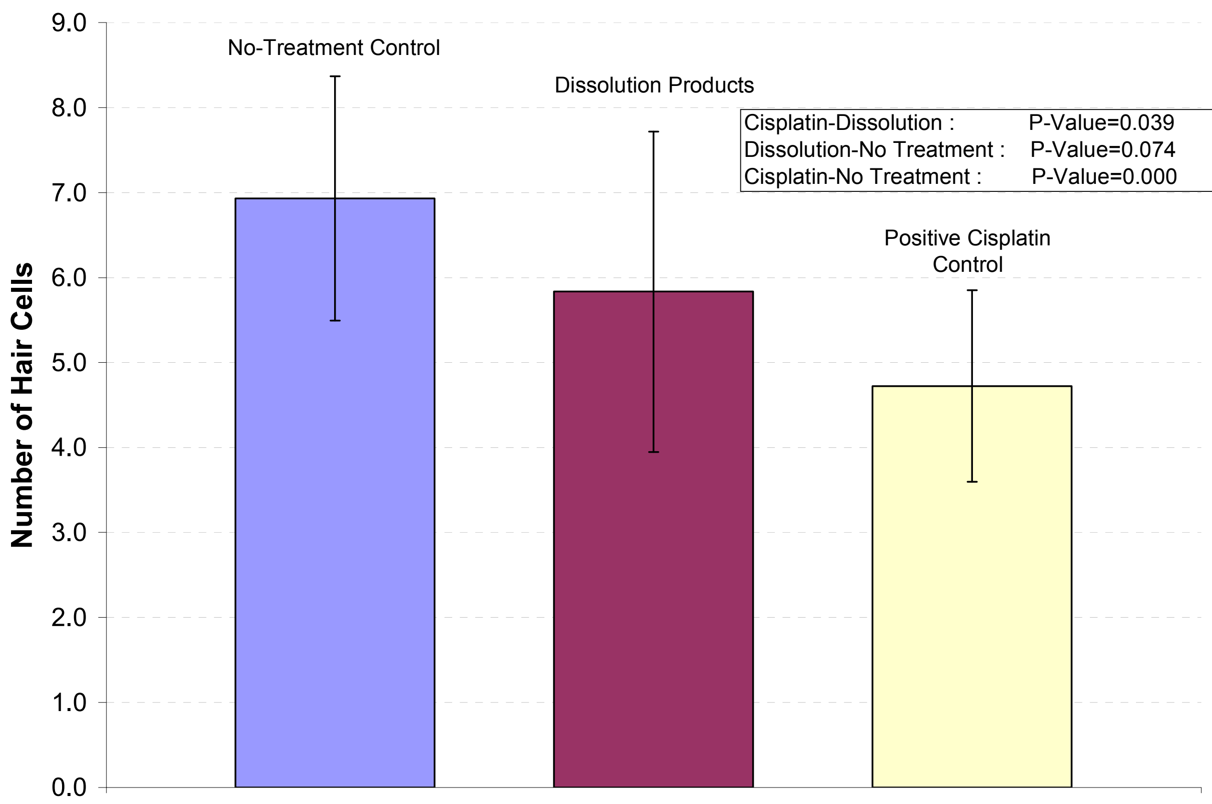


Figure 5-24: Hair cell viability (number of hair cells per neuromast) based on treatment. (n=18)

CHAPTER 6
DISCUSSION

Specific Aim 1: Development of a Calcium Alginate Tympanostomy Tube

Developing the Alginate TT

The first step in the development of an implantable alginate TT was to create gel tubes with the same geometry as commercial TTs. In order to achieve this, alginate tubes were initially constructed with considerably larger dimensions. This was due, in part, to the fact that during the drying and strengthening process alginate tubes shrink and loose up to 80% of their initial bulk. This shrinking is caused by the removal of water from the gel matrix and was controlled by the drying conditions. Tubes dried at 37°C (rather then 25°) immediately after manufacture, shrink rapidly, rendering them useless for experimentation. These tubes lose greater then 95% of their initial dimensions and are extremely difficult to remove from their glass support rods, without tearing or cracking. Furthermore, manufacturing tubes from smaller initial dimensions resulted in considerably thinner tubes, which may be incapable of withstanding the riggers of implantation or insertion. The optimal initial conditions that produce consistent and reliable results were those with a barrel diameter of 4.4mm, wall thickness of 1.6mm, and an overall length of 9.0mm. This resulted in a dried tube dimension of ~1.1-1.2mm inside barrel diameter (60% reduction), 0.1-0.2 mm wall thickness (80% reduction), and an overall length of ~6.0mm (33% reduction).

Similar dimensional constraints were applied to the Reuter Bobbin tubes prior to drying. The initial barrel diameter was set to 4.0mm and the shaft length was set to 3.0mm. The final dried dimensions were 1mm inner diameter and 2mm shaft length. Furthermore, with Reuter Bobbin tubes, it was important to maintain the separation distance between the two flanges (Figure 5-4, 5-5). These flanges provide a location for the latex membrane to reside during

testing, and maintained the geometric constraints necessary to allow comparison between commercial stainless steel Reuter Bobbin tubes. Results have shown that during flange repair, additional CaCl curing solution re-crosslinks broken gel components back into a solid. Therefore, flanges that were in contact with each other prior to CaCl application can become permanently fused preventing the latex from sealing around the barrel. As a result, care was taken, prior to desiccation, to separate the flanges of the tube using the back of a sharp blade (Figure 5-4, 5-5).

Furthermore, the addition of CaCl to the gel prior to drying served two purposes. One purpose was access to additional CaCl, which helped crosslink the remaining alginate within the gel. The second was that the extra moisture allowed the gel to dry at a slower rate producing a more flexible (elastic) gel tube. Preliminary experiments were conducted without the addition of any solutions prior to drying. The resulting tubes were very rigid and difficult to remove from their glass support rod. It was then discovered that perhaps the tubes were "too dry" resulting in very fragile brittle tubes. Therefore, additional liquid (in the form of water) was added to the tube and it was discovered that the resulting tubes were more elastic and could more easily be removed from the support rod. Further investigation yielded gels with higher concentrations of Ca that were often softer and had a more elastic consistency, making them easier to manipulate and insert. Therefore, CaCl solution was later substituted for water to help maintain mechanical strength, flexibility, and crosslinking.

Maximizing Mechanical Strength

Alginate:PEG Ratio: The four most important parameters associated with developing a reliable tympanostomy tube are mechanical strength, smooth geometry, dissolution, and biocompatibility. In designing our alginate tube, the primary focus was on maximizing mechanical strength. We achieved this by first drying the tubes over extended time periods and

by adjusting the alginate concentration. The results show (Figure 5-6 (tension experiments)) that as the alginate ratio doubles from 1 to 2 to 4 parts per sample the mechanical strength increased by ~40% and again by ~ 50%.

A similar trend was seen in Figure 5-8 for tube compression testing. However, the rate was not as consistent. Compression tests show a considerable increase in strength (~70% increase) as the alginate concentration increases (1 part to 2 parts) with a more gradual increase (~ 25% increase) in strength from 2 parts to 4 parts. Increased alginate concentration results in an increased crosslink density and higher solid volume fraction which increases gel stiffness in a fairly predictable manner [53]. PEG was added to the gels based on preliminary experiments using 100% alginate. Gel tubes manufactured using just alginate were very difficult to remove from the glass support rods, and were not very elastic. PEG provided a way to predictability decreasing the mechanical strength of the gel tubes using biocompatible components.

Ca Concentration: The calcium concentration used to crosslink the gel also plays an important role in mechanical strength with increased concentrations resulting in decreased strength. This goes against our initial assumption of a mechanical increase as Ca concentration increases [49]. Initially it was seen (at time = 0) that the higher Ca gels appeared stronger resulting from rapid crosslinking (high crosslink density) occurring at high Ca concentrations. Rapid crosslinking traps larger quantities of water within "fresh" gel tubes increasing strength initially. This has been observed in other studies showing increased tendencies of water molecules to diffuse into gels with high crosslinking density [53]. However, as the tubes are dried the water content within the gels dictates their final mechanical strength (Figure 5-7 and 5-9). Therefore, as the Ca concentration doubles, the mechanical strength decreased by ~55% from [0.5M] to [1.0M] and then ~80% from [1M] to [2M]. Water content within the gel may reduce the solid

volume fraction which may contribute to the decrease in mechanical strength. Compression results follow a similar, although less substantial trend with a ~25% decrease from [0.5M] to [1M] and ~50% decrease from [1M] to [2M].

Tube Drying Time: The compressive strength of the alginate gel tubes were all significantly greater then the silicone control, independent of drying time (Figure 5-9). This is due to the initial (non-dried) wall thickness of the alginate gel tubes. After crosslinking and before complete drying the wall thickness was more then 6 times greater then the silicone control which contributed to the increase in the compressive strength seen within the first few days (3 days).

Material Properties (Physical Characteristics): Previous studies have labeled alginate gels as viscoelastic resulting from crosslinks and entrapped entanglements contributing to its elastic behavior and other physical characteristics contributing to viscous behaviors (such as slippage) [39]. However, due to the aforementioned drying process, it is reasonable to believe that the alginate gels have a very low or no viscous component and are considerably less elastic than fresh crosslinked gels used in the above study. Therefore, numerical solutions for tension and compression calculations should assume the alginate gels to be elastic, isotropic, and symmetric materials.

Furthermore, the calcium alginate gels' physical appearance changes as a result of ingredient concentration, with lower CaCl concentrations producing "brittle-dry" white gels and high CaCl concentrations producing "clear-elastic" gels. This clarity could be the result of the additional water entrapped within the gels produced with high CaCl concentrations. Also, as the alginate ratio decreased, the gels became more fragile and difficult to handle due to less solid volume fraction and crosslink density.

In addition, the magnitude of the tension and compression results highlight the various testing methods. Tension experiments load the gel samples to failure and are therefore considerably higher (several orders of magnitude) than the compression tests which are a non-destructive experiment measuring functional compression. Therefore, it is important that mechanical comparisons be made only among experiment types.

Moreover, disparities between experiments of similar ingredient components and drying times (Alginate:PEG/CaCl concentration) can be attributed to drying variability. As shown in Figure 5-6 the average modulus for [0.5M] CaCl gel (Alginate:PEG [4:1]) dried for 7 days was ~50,000kPa. However, during the extended drying experiments (Figure 5-7) the modulus increased to ~100,000kPa after drying for ~7 days. Similar although less substantial differences were seen among tube compression experiments. Figure 5-8 shows [0.5M] CaCl gel (Alginate:PEG [4:1]) dried for 7 days with a compressive strength of ~300kPa whereas extended drying experiments (4-30 days) (Figure 5-9) show a compressive strength of ~250kPa. One explanation for this is attributed to large standard deviations due to fluctuations in the drying rate of the gels within the incubator. Furthermore, the testing environment (temperature/humidity) changes from day to day, which may have some affect on the mechanical properties of the gel. Although, considerations were made to normalize experimental conditions, it was not possible to completely control all aspects of testing due to equipment limitations. One solution, in the future, would be to perform all experiments within a temperature controlled environment such as an incubator.

Mechanical Strength Limitations: There are, however, tradeoffs associated with maximizing gel tube strength, including a possible reduction in dissolvability. Higher alginate ratios within the gel decrease the dissolution rate (Figure 5-14) and can result in incomplete tube

removal. Furthermore, tube strength is directly related to geometry, with thick walled gel samples having superior strength to thin walled ones. Once again there is a tradeoff in that thick walled tubes can create larger tympanic perforations within the eardrum, allowing pathogens to aspirate into the middle ear space. This can lead to secondary infection and further complications. Therefore, these parameters must be optimized to promote healing and in some cases can be specifically developed based on the patients needs.

Physiological Mechanics

Alginate tube compressive strength after agar encapsulation

Tubes encapsulated within an agar substrate, simulated conditions similar to in-vivo tissue encapsulation environments. Due to the high water content of the agar during the first 20-25 days, a reduction in compressive strength of the alginate gel was seen. As shown before, (Figure 5-7, 5-9) water within the matrix of the gel reduces compressive strength. After about 20-25 days, the moisture within the agar had decreased (via evaporation), allowing the 30 day compression results to increase. However, due to the lack of moisture it was impossible to remove the gel tube from the agar (after 30 days). Therefore, water was added to the agar gel, after 30 days, in order to soften the gel prior to tube extraction. However, after a 1 hour submersion, the resulting compressive strength was still statistically similar to the non-encapsulated control. Furthermore, all treatment results were more then 5 times stronger then the commercial silicone control tubes indicating agar encapsulation (wet or dry) has little effect on gel compressive strength.

Based on the drying of the agar gel over the length of the experiment, alterations were considered to amend the agar drying experimental protocol. However, the application of water to the agar every few days (preventing drying) was rejected because increased moisture would artificially reduce compressive strength. Therefore, it could not be determined if moisture

application or agar encapsulation was contributing to compressive loss. As a result, the agar encapsulation protocol was not altered over the 30 days and the gel was allowed to naturally dry over time. On the other hand, studies have shown that mammalian cells may not readily interact with alginates due to the absence of cell-surface receptors and lack of protein absorption [54] limiting the development of complete tissue encapsulation in-vivo.

Alginate tube compressive strength after exposure to ototopical solutions

Over the lifetime of tube implantation it is reasonable to believe that various solutions will come in contact with the tube (other then the dissolution solution) resulting in premature dissolution or weakening. The mechanical strength of commercial silicone tubes were used as a minimum threshold for mechanical stability. Alginate gel tubes with a compressive strength below silicone were considered non-functional. The results show that all solutions caused a significant decrease in strength, compared to the no treatment alginate control (Figure 5-11). However, none of the solutions resulted in a decreased strength below the commercial silicone tube. In fact, all treatments were more then 50% stronger then the commercial silicone tube (after 24 hours). In addition, unlike the majority of the tested solutions, ear mucus and blood serum are unique, given that they may have prolonged tube exposure resulting from the healing process. Therefore, significant loss in compressive strength from either solution can be used as an indicator of long-term performance. However, both blood serum and ear mucus showed a loss in compressive strength of 47-58% respectively (well above that of the silicone control). This is despite the need to submerge the ear mucus tube prior to compression testing within a PBS solution for 1 hour. Ear mucus over the 24 hour exposure period (at 37°C) dried around the tube and created a crust, preventing removal. PBS was added (1 hour soak) to remove the tube from the mucus without damage. Even with the re-introduction of "water", the ear mucus tube

lost only 58% of its initial strength and was still more then twice the strength of the commercial silicone tube.

Furthermore, upon contact with the tube, it was observed that soap water created a milky white solution. However, it proved to have very little effect on the overall strength after 24 hours. Antibiotic solutions, specifically Ofloxacin, showed a 73% decrease in mechanical strength after 24 hours, making it the most detrimental of the solutions. However, the resulting gel was still ~50% stronger than the commercial tube. It is important to note that for each experiment, the tubes were completely submerged in their respective solutions, simulating a worst case scenario. In-vivo conditions will most likely not provide complete 360° submersion and may potentially limit mechanical loss. Furthermore, although significantly lower than the alginate control, all the alginate gel tubes have the potential to re-dry after ototopical application, regaining strength.

Minimizing plugging

Tube plugging is one of the most prevalent complications associated with TT implantation. Tubes manufactured with very smooth inner lumens have decreased occlusion prevalence. As a result, alginate tubes were developed on glass rods providing a smooth inner diameter. Also, previous studies have shown that higher concentrations of alginate within the gel matrix produces a smoother surface [55], further validating the use of high alginate concentrations when developing tubes. This smooth lumen prevents mucus (egg white) from adhering to the inner surface of the tube and, therefore, allows fluid to extrude into the external ear canal rather then building up on the inner surface of the tube.

HSA Application: Further tube enhancements included the addition of HSA to reduce mucous adhesion. HSA coatings indicate that it may be possible for microscopic imperfections in the surface of the tube to increase resistance and facilitate plugging (Figure 5-12). HSA

coating provides a very thin barrier to adhesion that can be applied prior to tube insertion [7,56,57]. However, HSA application involves the submersion of the tube, without its glass support rod, in a liquid solution (Phosphate Buffered Saline (PBS)) containing HSA for 12 hours. This submersion re-hydrates the gel tube which, in tern, decreases its mechanical strength. Nevertheless, this loss in mechanical strength appears minimal, having little to no effect on the manipulation and insertion of the alginate tube into the latex membrane for testing. Furthermore, removal of the glass rod permits coating of the inner barrel surface with albumin. However without its glass support, it was noted that the tube lumen shrank due to swelling of the gel. This swelling can artificially increase the occlusion rate by decreasing the tube diameter, while limiting comparisons with commercial tubes. As a result, after submersion each tube was re-inserted onto its glass rod to re-form the lumen of the tube and maintain its uniform circular state. During insertion, care was taken to not tear the gel or to artificially enlarge the tube opening using the glass rod.

Occlusion results were compared between both un-coated tubes and to albumin coated alginate tubes in order to determine if albumin coatings reduce the occlusion rate. It was shown that un-coated alginate tubes have a reduced occlusion rate when compared to un-coated commercial stainless steel tubes. This can be partially attributed to the possible decreased adhesion properties of the mucus simulant to the alginate gel. Based on these results it was not surprising to see a further reduction in occlusion rate with the application of albumin. Albumin coated alginate tubes showed a 50% reduction in occlusion due to the decreased resistance provided by the albumin.

Moreover, HSA coated/un-coated tubes were tested immediately after PBS submersion and were not dried prior to experimentation. It is unknown what affect moisture will have on

occlusion rates. However, previous experimental protocols [7,56,57] did not include drying after albumin application. Despite this, throughout the 2 hour occlusion experiment, the alginate gels did dry somewhat (visually) and after experimentation appeared to have regained most of their original strength.

Specific Aim 2: Optimize the Gel Dissolution Components for Complete Dissolution

The ability to dissolve the gel tube after implantation is one of the novel features of our TT design. Prior research studies have focused on the use of absorbable tubes that dissolve at a set rate independent of physiological conditions. Therefore, once inserted there is no control over absorption. Our tube design provides the ability to dissolve the tube using a dissolution solution which affords extra control to the physician and alleviates the need to "guess" the absorption time upon insertion. Dissolution is critical to promoting long term healing, since prolonged tube implantation has been shown to be detrimental [2]. However, it is not necessary to sacrifice mechanical strength for rapid dissolution. Dissolution time frames of around one week may be acceptable in order to limit ototoxicity and maintain mechanical strength. Furthermore, the use of a minimally invasive dissolution technique (solution injection vs. surgical removal) further reduces trauma to the patient and promotes natural healing.

Effect of Various Dissolution Solutions on Dissolution Rate

Complete tube dissolution was limited to contact with dissolution solutions and not exposure to ototopical solutions. Dissolution is thought to occur when calcium within the calcium alginate gel is replaced by Na ions available from the dissolution solution, remaking sodium alginate solution. Three dissolution solutions were tried with the intention of optimizing the dissolution rate of the gel. Optimal tube dissolution consists of complete removal of the tube with little physical remains. However, as shown in the results (Figure 5-14 5-15), material properties (gel tube composition) greatly affect the dissolution rate and complete dissolution

cannot be achieved in every case. The three solutions used for our experiments were NaCl, Vinegar and $NaHCO_3$. The only two dissolution combinations that resulted in any noticeable changes in the tube removal were NaCl + $NaHCO_3$ (50:50 mixture) and $NaHCO_3$ alone. Both solutions showed considerable affinity for dissolving [1:2] Alginate:PEG gels crosslinked with [0.5M] CaCl. Dissolution was achieved by the re-introduction of Na into the matrix of the gel at a higher concentration than Ca present in the gel, which increased swelling and promoted dissolution [39].

Furthermore, prior to the use of $NaHCO_3$, NaCl, and vinegar, several other dissolution solutions were tried with minimal success including sodium phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) (result: soft, denatured tube), potassium chloride (result: soft, denatured tube), sodium hydroxide (result: soft, denatured tube), magnesium sulfate (result: soft, denatured tube), potassium hydroxide (result: almost complete tube dissolution) and sodium sulfate (result: almost complete dissolution). In each case, the tubes remained in tact after several hours with the exception of potassium hydroxide and sodium sulfate. These two solutions showed promising dissolution but could pose ototoxicity concerns. In the end, there are limitless possibilities for additional dissolution solutions however, very few if any have been previously tested for ototoxicity and therefore should be used with caution.

In addition, the dissolution solutions used to remove the solid gels (NaCl + $NaHCO_3$ and $NaHCO_3$ alone) play an important role in tube removal. However, the rate of dissolution across all tube types using NaCl + $NaHCO_3$ was not significantly greater than $NaHCO_3$ alone and did not justify its continued use. Initially (within one hour), $NaHCO_3$ appears to dissolve the gels faster than those solutions containing NaCl + $NaHCO_3$. Furthermore, vinegar, along with $NaHCO_3$, did produce a vigorous reaction (CO_2 gas). However, the reaction was not sufficient

enough to last longer than a few seconds and did not facilitate degradation of the tube. It is possible, though, that after degradation has begun, a vinegar solution can be applied to break up larger gel pieces and achieve compete dissolution.

NaCl alone showed very slow dissolution. Furthermore, over 24 hours there appears to be no change in the tube submerged in NaCl. This phenomenon has been confirmed by others [39] dissolving alginate gels with NaCl. Immediately after submersion (NaCl several hours), mechanical strength of the dried gel tube is decreased due to gel hydration and swelling. However, after that initial time period no further degradation was achieved (up to 1 week [39]). In addition, the NaCl solution used was of relatively low concentration (0.85% w/v or 0.303M). Based on a weight per volume ratio there was more NaCl (1.515×10^{-4} mol) than calcium alginate (9.09×10^{-5} mol) present during dissolution (Appendix E). Therefore, NaCl should be capable of dissolving alginate gels. However, a difference of 6×10^{-5} mol (or 1.6x) appears to not be sufficient enough to result in rapid dissolution. Literature has also confirmed that degradation of low α-L-guluronic acid alginates (M/G ratio > 1.0, our alginate M/G ratio = 1.6) can be prevented by submersion in solutions with a Na^{+}:Ca^{2+} ratio of 3:1. The amount of NaCl relative to calcium alginate is less than 3:1[45] (1.515×10^{-4} mol vs. 9.09×10^{-5} mol) preventing dissolution.

An additional factor that may contribute to dissolution includes byproduct solubility. The dissolution of the alginate gel using NaCl may result in the production of CaCl as a byproduct. The solubility of CaCl (74.5g/100ml, 20°C) is greater than that of $CaHCO_3$ (calcium bicarbonate) (16.6g/100ml, 20°C). Comparing the solubility of the possible dissolution products $CaHCO_3$ vs. CaCl there is support for higher dissolution rates with $NaHCO_3$. The production of $CaHCO_3$ precipitate is less soluble in aqueous solution, thus driving the reaction in favor of

$NaHCO_3$ dissolution over that of NaCl. CaCl is soluble in H_2O therefore, Ca ions are available in solution to re-crosslink the alginate gel limiting disintegration. On the other hand $CaHCO_3$ precipitates out of solution at relatively low concentrations removing Ca from solution and promoting disintegration. This supports results that the $CaHCO_3$ shows faster, more complete dissolution than the NaCl.

Furthermore, other factors can come into play, including the relative pH of the solution, and porosity of the polymer surface. Alginate gel porosity can be reduced via desiccation. Furthermore, pore size can be further reduced in the presence of low pH solutions. NaCl has a pH of ~7.0, whereas $NaHCO_3$ has a pH of 8.6. This small change in pH may potentially limit rapid dissolution when using NaCl. As a result, the reduction in pH, along with solubility constraints and a Na:Ca ratio below 3:1, may combine to limit degradation via NaCl submersion. Based on these results it appears that it is not just the Na present in the solution that drives dissolution, but rather a combination of factors leading to tube removal.

Gel Tube Composition: Results show that gel composition plays a vital role in the rate of dissolution. Gel compositions tested for dissolution represent the max and min (extreme) values of gel composition and were used to obtain values of the max and min dissolution rates for various tube compositions. Gels such as those composed of [1:2] Alginate:PEG crosslinked with [0.5M] CaCl showed an affinity for dissolution (R=4.6). However, gels composed of similar Alginate:PEG ratios [1:2] but with higher [2.0M] CaCl curing solutions has slower dissolution rates (Figure 5-14 5-15). This can be explained by the excess CaCl remaining within the [2.0M] crosslinked gel, which is capable of maintaining the high crosslinking density necessary for gel formation. As expected, it appears that over time (1-5 hours), tubes composed of low alginate concentrations and low CaCl concentrations had a higher dissolution affinity than all other

combinations. Although slower in terms of dissolution, [4:1] Alginate:PEG crosslinked with [0.5M] CaCl has superior mechanical strength when compared to all other gel combinations. Therefore, the extra increase in dissolution time (generally >5 hours using 0.5M $NaHCO_3$) was justified over the long implantation time. All other tube combinations (after 5 hours in $NaHCO_3$) achieved an average dissolution value of R=4 which is quite respectable considering their varying composition and mechanical strength.

Effect of $NaHCO_3$ Concentration on Dissolution Rate

Results show that the concentration of $NaHCO_3$ in solution does affect the rate of dissolution. However, the results were not linear; rather dissolution peaks at 0.5M followed by decreases in dissolution at 1.0M and 2.0M (Figure 5-16). At a concentration of 0.1M, the amount of $NaHCO_3$ (5×10^{-5} mol) present in solution was less than the amount of alginate (9.09×10^{-5} mol) in solution. Therefore, dissolution should be limited. However, degradation progressed slowly (requiring ~24 hours to complete). According to the literature [39,45] $NaHCO_3$ dissolution should be limited due to low Na availability along with a Na^+:Ca^{2+} ratio of < 1 (described above). Therefore, other factors may come into play, such as pH (8.6 vs. NaCl 7.0) along with the possible development of secondary byproducts such as $CaHCO_3$. The increase in pH may facilitate an increase in gel porosity, which may promote dissolution using 0.1M $NaHCO_3$. Furthermore, the development of $CaHCO_3$ precipitate, with its low solubility, may provide additional bonding sites for Ca during dissolution. However, due to the relativity short longevity of $NaHCO_3$ solution, it may be more efficient to re-apply the 0.1M solution several times to enhance its effect.

On the other hand, dissolution using 0.5M $NaHCO_3$ contained approximately twice as much $NaHCO_3$ (2.5×10^{-4} mol) as alginate (9.09×10^{-5} mol) and appears to dissolve alginate gels rapidly. The ratio of Na:Ca present in solution was ~3x which is necessary for alginate

dissolution. Moreover, very high concentrations of $NaHCO_3$ (1.0M, 2.0M) (5×10^{-4} mol and 1×10^{-3} mol vs. Alginate at 9.09×10^{-5} mol) approach the saturation point of $NaHCO_3$ in solution (10g/100ml at 20°C). Therefore, Na is precipitated out of solution and is not available to replace Ca within the alginate gel (Appendix E). However, since one of the goals of this project was to minimize ototoxicity, it is preferable to use lower $NaHCO_3$ concentrations for experimentation.

Physiological Dissolution

Two methods were used to dissolve the tube: complete submersion (within a 24 well plate) and physiologic dissolution (within an in-vitro apparatus). Complete submersion (0.5-1.0ml solution) provided total envelopment of the gel tube and promoted uniform dissolution. This represents the "best case" for dissolution because the entire tube is dissolved at one time. However, this method was not possible after in-vitro tube implantation. Therefore, a physiological method was developed that simulates conditions the clinician will see during dissolution (access to only one side of the tube via external ear canal). Using this method, tube dissolution was achieved first with consumption of the external "flange". Over time, the barrel portion of the tube was degraded to the point where it released from the flange and was dissolved within the holding well. Dissolution results using both methods were similar, with both techniques achieving complete dissolution within parallel time frames (Figure 5-16 and Figure 5-17).

However, physiological dissolution appears to have a slight edge due to separation of the thinner barrel portion from the thicker flange portion. During the first hour, dissolution rates were slower (R~3.2) for the flange portion of the tube due to increased gel thickness. However, the barrel portion of the tube dissolved rapidly, sometimes within one hour due to its small size and minimal thickness. This is a consequence of the manufacturing process, which produces gel

tube flanges more than two times thicker than the barrel portion, resulting in longer dissolution times. After 5 hours both the barrel and flange portions of the tube had dissolved to R=5.5.

Although the flange portion takes longer to achieve complete dissolution, its location during testing was designed to maximize dissolution time. Knowing that the flange dissolves slower than the barrel its placement within the in-vitro ear chamber was inverted (from in-vivo) in order to present the thicker portion for dissolution. In this way the maximum dissolution time can be determined for tubes with thick external flanges.

Although all in-vitro experiments were performed with the flange portion of the tube facing towards the external ear canal, in-vivo implantation requires that the flange face medially preventing premature extrusion. Therefore, based on our results, in-vivo dissolution times may be enhanced within the middle ear space. On the other hand, the thinner barrel portion can dissolve rapidly within the external ear canal (in-vivo), allowing the thicker flange to fall into the middle ear space to begin dissolution. As a result, after in-vivo implantation and subsequent dissolution, it is the thick flange portion of the tube that will contribute to any ototoxic effects. However, dissolution and perhaps ototoxicity can be reduced with the development and subsequent dissolution of smaller more uniform TTs. In the present experiments, all dissolution experiments were performed using T-shaped tubes which can have barrel lengths of up to 6 mm and thick flanges. Larger tubes were used to determine the maximum time (worst case) for dissolution.

Effect of desiccation on dissolution

Dehydration of the gel tube removes moisture and creates a strong rigid tube. Alginate TTs were designed to be implanted for extended time periods (months/years) and moisture loss will continue as implantation time progresses. The results show that throughout drying, the rate of dissolution increases, achieving complete dissolution. Tubes dried for only 7 days achieved

complete dissolution after only 5-24 hours (depending on tube/dissolution solution ingredients) (Table 5-1). However, tubes dried for extended time frames (15-30 days) showed a marked decrease in dissolution time (1-2 hours using 0.5M $NaHCO_3$) (Figure 5-17). This may be the result of the reduction in surface area (80% smaller dried tubes) compared to fresh tubes. Furthermore, the porosity of fresh tubes is most likely larger than that of dried tubes (drying decreases porosity [45]), but fresh tubes are significantly thicker than dried tubes. The increased size of "fresh" tubes may prevent Na from rapidly penetrating the gel and replacing Ca during the dissolution process. Therefore, fresh tubes dissolve slower.

Effect of HSA coatings on dissolution

Tubes coated with HSA limit occlusion formation and have been extensively studied in literature [7,56-58]. In addition, tubes coated with albumin appear to dissolve within 5 hours posing no negative affect on dissolution (Figure 5-18). These results can be linked to the albumin coating process, which involves submerging the tube within a PBS solution containing 0.01% HSA. This application process allows sodium along with potassium ions, located within the PBS solution, to begin to break down the tubes prior to formal dissolution. However, uncoated alginate tubes submerged in PBS show a slight decrease in dissolution, implying albumin application may aid dissolution as well. One limitation of this technique was that, prior to experimentation, the albumin coated tubes were not dried, which may artificially increase the performance (dissolution/occlusion) of the tube.

Removal of retained tube components

After application of the dissolution solution it is possible for part of the tube to remain un-dissolved. Un-dissolved components (along with broken tube parts) within the ear canal can be retrieved by the clinician via the external ear canal. However, components of the tube within the middle ear space can not be physically removed and will become absolved over time. The

biocompatible nature of the gel inhibits the immune response and over time, via cellular encapsulation and degradation, the warm moist environment of the middle ear will absolve the remaining alginate gel components. The timeframe for complete tube dissolution should be limited to within one week. Prolonged dissolution times (> one week) may lead to the development of secondary complications.

Specific Aim 3: Quantify Ototoxicity from Exposure to Dissolution Products

Biocompatibility associated with TT dissolution can be related to Zebrafish ototoxicity. Ototoxicity was minimized by manufacturing TTs from ingredients previously used in medial implants and with the use of dissolution solutions chosen from clinical ototopical treatments.

Across all parameters (fluorescent intensity, neuromast area, and number of viable hair cells) dissolution products outperformed known ototoxic treatments (Cisplatin). There were no significant differences between the no-treatment (negative control) and those fish exposed to the dissolution products. However, a decreasing trend was seen between the two groups (Figure 5-22, 5-23, 5-24). This declining trend could be the result of the increased salinity of the dissolution products. However, effects can not be isolated between dissolution products and salinity based on the Zebrafish model limitations. Mammalian experiments with a higher threshold for salinity will need to be conducted in order to isolate individual effects.

Zebrafish were used to confirm the viability of hair cells after exposure to dissolution products. However, due to tonicity limitations (fresh water fish in a salt water environment), Zebrafish embryos could only be maintained within a 0.1M $NaHCO_3$ solution for 30 minutes. Mortality associated with longer exposure times (>30 minutes) was accelerated due to high concentrations of $NaHCO_3$ within the dissolution product. This concentration (0.1M) was more than 20 times greater than the $NaHCO_3$ concentration found in embryo media (extreme testing

condition)(Appendix E). Therefore, any ototoxicity associated with the dissolution solution can be partially attributed to the high salinity of the water (Zebrafish model limitation) and not necessarily the dissolution products themselves. Along with the increased sensitivity of Zebrafish and extreme testing conditions ototoxicity was still minimized after exposure to dissolution products. This indicates the potential viability of using dissolvable alginate gels as an alternative to commercial tubes.

While a direct correlation could not be made between Zebrafish ototoxicity and dissolution (30 minute exposure vs. 24 hour exposure at 0.1M $NaHCO_3$), previous clinical studies have provided a link. It has been shown that (Appendix E) $NaHCO_3$ is the primary component within the dissolution solution making up more then 20 times the volume of the dissolution products. Therefore, it may be possible to assume that $NaHCO_3$ is the primary ingredient driving potential ototoxicity. As a result, previous studies [22,23,25] have shown $NaHCO_3$ use (to relieve obstructed tubes) at concentrations up to 5% or ~0.59M. Although minimal pain was associated with in-vivo application [23] hearing loss was not attributed, indicating $NaHCO_3$ efficacy in humans up to 0.59M. At this concentration, ototoxicity is not anticipated and dissolution was achieved within a short time frame (1-2 hours). Further testing using mammals (ex. guinea pigs) will have to be conducted to measure ototoxicity at higher $NaHCO_3$ concentrations.

pH may also be an issue associated with Zebrafish ototoxicity. However, results show that pH remains constant (Table 6) as $NaHCO_3$ concentration (in embryo media) is increased from 0.001M up to 2M. Furthermore, previous studies have also shown that Zebrafish can survive within solutions as high as (pH 8.9) for up to 15 days [59].

Furthermore, osmotic affects associated with $NaHCO_3$ injection may lead to hearing loss in mammals. Increasing the sodium bicarbonate concentration can lead to fluxuations in the osmotic conditions within the lymphatic system of the cochlea. These alterations, between the perilymph and endolymph fluids, can affect the pressure within each system. Increased pressure results from an osmotic imbalance of salts (potassium (K) and/or sodium (Na)) which may cause fluid to be drawn into the perilymph (extracellular fluid) pushing the basilar membrane (separates scala tympani and scala media within inner ear) towards the scala tympani (perilymph cavity within the cochlea). This movement of the basilar membrane can affect the electrophysiology of the cochlea resulting in decreased hearing [60]. However, the method of entry into the cochlea after tube dissolution is thought to occur through the round window (middle ear) which is selectively permeable. It has been shown that round window permeability can be decreased in the presence of overlying residual effusion, the presence of granulation tissue, or thickening of the round window membrane which can all be present in infected ears. Permeability also appears to be a function of concentration as seen with HSA levels, which are impermeable below a minimum concentration. Therefore, if the concentration is below the threshold value then the toxic chemicals may not be able to enter into the inner ear to cause damage. Furthermore, upon entering the round window the concentration of the chemical must be high enough to stimulate biochemical or immunochemical reactions. Therefore, ototoxicity relies on several factors combining to cause hearing loss and as a result may be minimized [61].

Table 6-1: Embryo media solution pH as $NaHCO_3$ concentration is increased from 0.01M-1.0M

Solution Concentration	pH
$NaHCO_3$ 0.01M	8.6
$NaHCO_3$ 0.05M	8.6
$NaHCO_3$ 0.1M	8.6
$NaHCO_3$ 0.5M	8.5
$NaHCO_3$ 1.0M	8.3
Embryo Media	7.2

CHAPTER 7
FUTURE WORK

Based on the results obtained from this report, additional experiments will need to be performed in order to implant alginate tubes into human patients.

Tube Manufacturing: Limitations including variability in geometry and dissolution times will need to be addressed prior to in-vivo application. The use of an enclosed injection mold for fabricating geometrically uniform tubes is necessary for the production of implantable tubes. Dissolution variability associated with differences in flange/barrel thickness can be eliminated using more sophisticated molds. Furthermore, uniform drying can be achieved via lyophilization (freeze drying) techniques, further increasing uniformity. Also, mechanical strength can be enhanced with the use of high G-block alginate gels (long length G-blocks), which result in more molecular entanglement and higher mechanical strength, although it may limit dissolution.

Experimental Recovery: Based on our results, it is unknown what permanent mechanical losses, if any, are associated with repetitive exposure to various solutions. Therefore, experiments will need to be conducted to determine if there are any lasting mechanical effects of repetitive exposure to ototopical solutions. Moreover, additional experiments will need to be conducted using other ototopical agents with potential exposure to the tube. These treatments can include (Sofradex [22], lipase, hyaluronidase [24], locorten vioform, and olive oil, [25] among others.)

Dissolution: Premature dissolution, resulting from in-vivo cellular/tissue encapsulation associated with alginate gel implantation can be limited with the application of poly-L-lysine-MPEG and poly-L-lysine. These coatings have been shown to limit protein absorption while decreasing cellular interactions and immunogenicity [45]. Furthermore, dissolution limitations,

including injection techniques, can be modified to facilitate long term dissolution. Dissolution application can be adjusted to include dissolution gels, which can maintain their position within the ear canal after implantation. Currently, dissolution solutions have a low viscosity and can become expelled during standing and walking. Dissolution is limited if contact is not made between the dissolution solution and alginate tube. Therefore, thick dissolution gels, such as those composed of Dextran can be infused with $NaHCO_3$ and applied to the tube. These gel solutions have the potential to maintain their position after insertion, decreasing treatment time. Furthermore, dissolution can be localized with the application of a gelfoam pledgette that can be soaked in $NaHCO_3$ solution and applied to the lateral surface of the gel tube. The delivery mechanics of gelfoam may allow the barrel/flange portion of the tube to dissolve leaving the remaining portion to act as a scaffold for tissue growth. This dissolution method may also limit $NaHCO_3$ from entering the middle ear space (reducing ototoxicity), and therefore, higher concentrations of $NaHCO_3$ can be used to dissolve the gel. After dissolution, the gel foam can be removed by the clinician. In addition, dissolution time may be reduced with the use of Reuter-Bobbin tubes with their 2mm shaft length and significantly smaller (weight and volume) dimensions. Further work can focus on optimizing the dissolution concentration to minimize dissolution time, while simultaneously minimizing ototoxicity in mammals and eventually humans.

Ototoxicity: Furthermore, additional ototoxicity experiments will have to be conducted first on small mammals followed by human clinical trials. Successful Zebrafish experiments indicate that the dissolution products have a minimal affect on hair cell function after 30 minute exposures. However, the limitations of Zebrafish as an ototoxicity model preclude their use in further experimental analysis. The high salinity produced by the sodium bicarbonate solution

provides a habitat that is inhospitable for Zebrafish testing over extended time periods. Although, good for rapid, inexpensive, qualitative results, Zebrafish ototoxicity experiments are deficient for optimizing dissolution time. Future experiments must be conducted using mammals (ex. guinea pigs) to determine if hearing loss is associated with implantation and removal of alginate gel TTs using higher concentrations and longer exposure times, approaching 0.5M and up to one week. Following this, clinical patient studies will have to be conducted to validate experimental results and to optimize components.

CHAPTER 8
CONCLUSION

Based on the results presented in this paper it can be concluded that it is possible to develop a calcium alginate TT. We have shown that the mechanical strength is superior to that of commercial tubes, providing reliable ventilation. Furthermore, these tubes can be removed from a simulated tympanic membrane with the use of a dissolution solution reducing the need for more invasive procedures. Finally, the resulting tube dissolution has been shown to have a minimal affect on hair cell function using a Zebrafish model. As a result, it may be possible to develop a biocompatible alginate TT capable of providing and maintaining reliable ventilation while dissolving on demand. Individual conclusions concerning the development of a biocompatible and dissolvable TT are summarized below.

1. Experiments have proven that the mechanical properties of calcium alginate gels exceed those of commercial silicone tubes.

2. In the presence of an agar substrate, mechanical superiority was maintained after in-vitro encapsulation for 30 days.

3. Exposure to ototopical solutions including ear mucus and blood serum proved to be detrimental. However, after 24 hours (complete submersion) the resulting compressive strength was still more then 50% greater then that of commercial silicone tubes.

4. When compared to commercial stainless steel Reuter Bobbin tubes, tube plugging was minimized with the use of alginate tubes (in-vitro). Un-coated alginate gel TTs showed a 20% decrease in plugging using an in-vitro ear chamber.

5. Mechanically superior alginate tubes can be completely dissolved with the use of solutions containing $NaHCO_3$, potentially reducing the need for more invasive surgical procedures.

6. Dissolution efficacy may be enhanced with increased drying time and remains unaffected by the application of albumin coatings.

7. Using a Zebrafish ototoxicity model, biocompatibility (hair cell viability) associated with alginate gel dissolution was maintained.

APPENDIX A
TENSION AND COMPRESSION TESTING FOR ALGINATE GEL SPECIMEN

Mechanical testing apparatus (tension) (Figure A-1, A-2, A-3) showing the “dog-bone” specimen as it is pulled to failure. Figure A-4 describes the compression of the alginate gel tube between two flat platens.

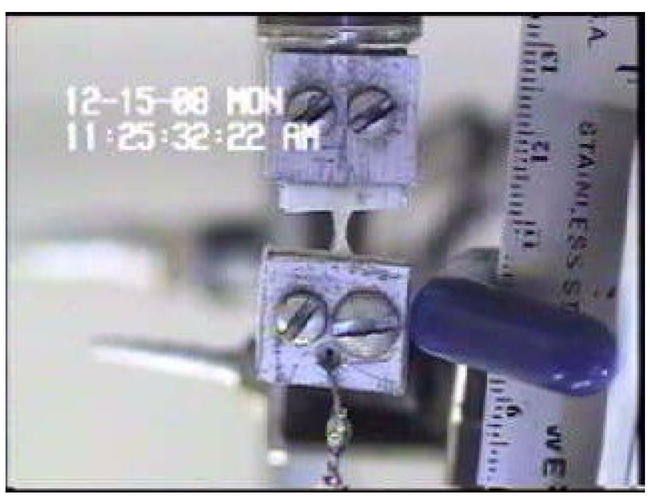


Figure A-1: “Dog-Bone” specimen in clamps (time=0)

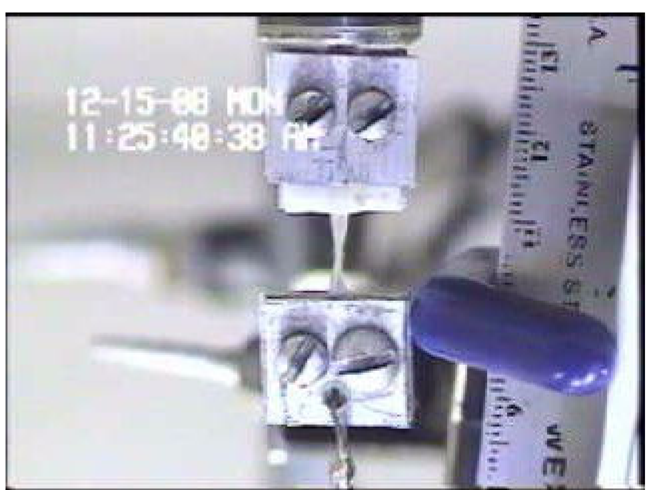


Figure A-2: Specimen pulled and elongated. Sample becomes thinner.

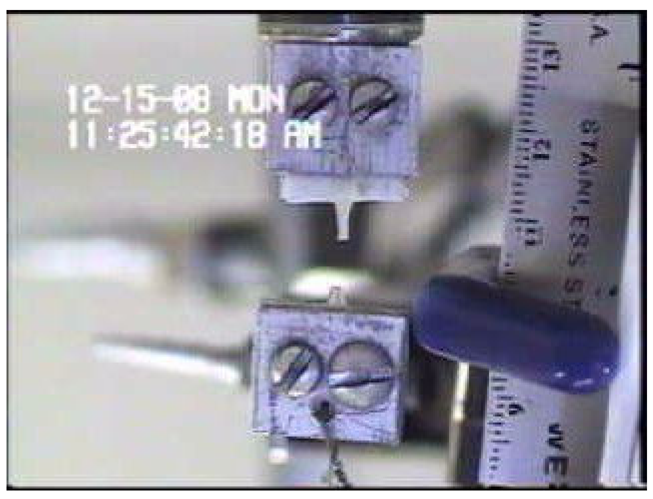
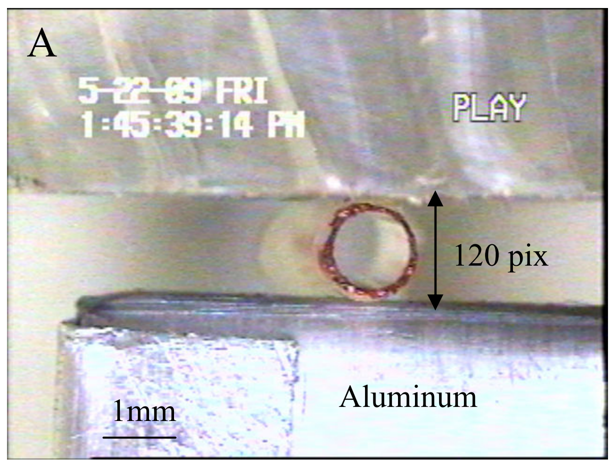


Figure A-3: Sample fractured with a clean even break.



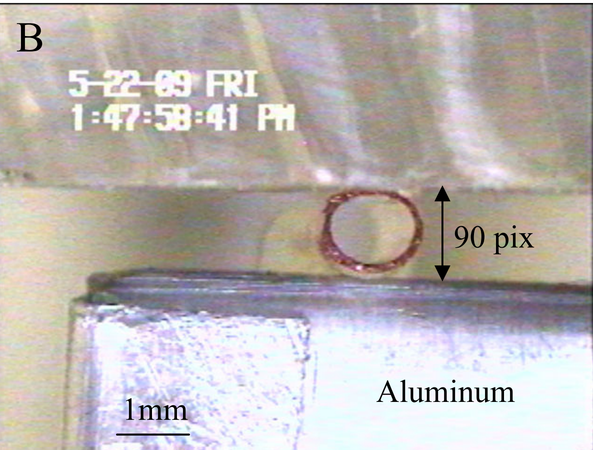


Figure A-4: Alginate gel tubes deformed between aluminum and PDMS platen. (A) Shows the un-deformed state of the gel tube placed between two flat platens. The top platen is composed of PDMS and the bottom platen is composed of aluminum. (B) Shows the deformed state (25% of initial diameter) after compression. (NOTE : Initial diameter measured prior to addition of the aluminum platen (A) ~120 pixels, final diameter (B) ~90pixels. Pictures are inverted due to camera mounting, bottom section represents the aluminum funnel).

APPENDIX B
NUMERICAL SOLUTION FOR TUBE COMPRESSION

Compression Deflection:

$$CD = \frac{F}{A} \quad \text{(B1)}$$

CD = compression deflection (compressive strength) force per unit of specimen area, (kPa) [46]. F = force required to compress the specimen 25% of the outer diameter, (N). A = specimen compression contact surface area, (m^2).

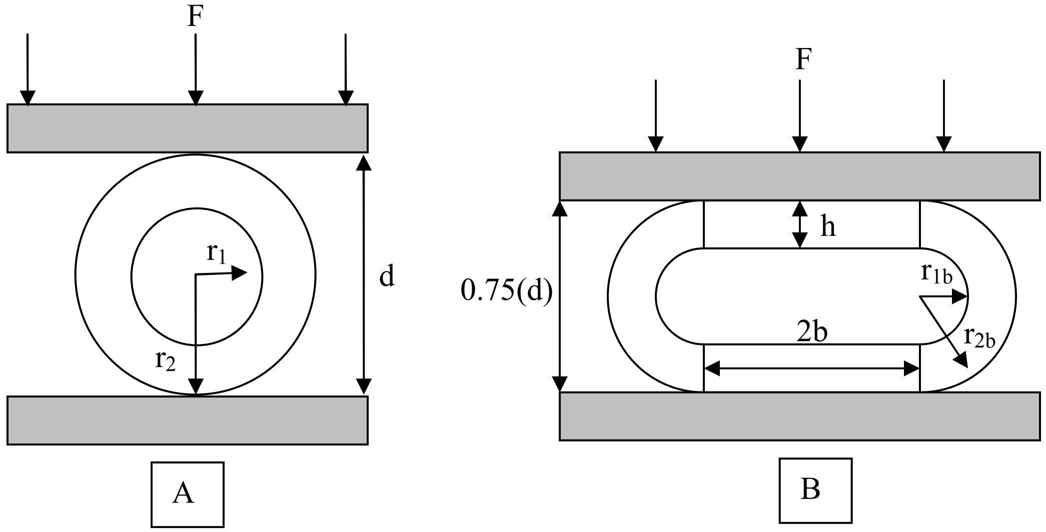


Figure B-1: Compression of alginate gel tubes between two flat platens. (A) Undeformed silicone and alginate gel tubes. d = distance between the two platens. r_1 = inner radius. r_2 = outer radius. F = force (load) applied to the barrel of the tube. (B) Deformed tube (25%) showing contact width of 2b. Two half cylinders with radius r_{1b} = inner radius deformed tube, r_{2b} =outer radius deformed tube, h = thickness of tube barrel.

Assumptions:

- Material is isotropic
- Geometry is uniform across all tube types
- Deformation is uniform/symmetric
- Wall thickness is constant
- Contact area is constant across all tube types

Based on the conservation of volume and the constraint that h = constant, the front surface area of the undeformed tube (Figure B-1-A) and the deformed tube (Figure B-1-B) should be equal, assuming the tube deforms symmetrically into two half circles separated by two rectangular contact sections. Since the geometry of the tubes are equal (independent of gel composition) and the tubes are all equally deformed by 25%. It is reasonable to assume that the contact area for all tubes is equal. The only change being the load (F) required to compress the tubes 25%. Therefore, the contact surface area (A) from the above equation was calculated using Equations B2-B5 at the time the load compressed the tube 25% (Figure B-1). L = length of contact the tube has with the bottom platen. 2b = the width of contact with the surface.

$$Contact\ Area\left(rec\tan gle\right) = 2b \times L \tag{B2}$$

$$Area\ of\ undeformed\ tube\ (A_U) = A_2 - A_1 = \pi r_2^2 - \pi r_1^2 \tag{B3}$$

$$Area\ of\ deformed\ tube\ (A_D) = 2(2b \times h) + 2\left(\frac{\pi r_{2b}^2}{2} - \frac{\pi r_{1b}^2}{2}\right) \tag{B4}$$

Where:

$$\begin{aligned} r_{1b} &= r_{2b} - h \\ r_{2b} &= \frac{1}{2}(0.75)(d) \\ h &= wall\ thickness \\ d &= tube\ \ outer\ \ diameter \end{aligned}$$

Setting (B3) equal to (B4) and solving for b gives:

$$\begin{aligned} b &= \frac{A_U - \pi(r_{2b}^2 - r_{1b}^2)}{4h} \\ b &= \frac{0.637mm^2 - \pi(0.5625mm^2 - 0.4125mm^2)}{4(0.15mm)} = 0.295mm \end{aligned} \tag{B5}$$

Force (F)

F = force required to compress the Alginate:PEG specimen 25%.

$$\text{Force} = \text{ma (m = mass, a = acceleration of gravity (constant))} \quad \text{(B6)}$$

m = 35.5g (average mass for calcium alginate, changes based on composition), g = 9.81 m/s^2

$$F = ma = (35.5g)(9.81m/s^2) = 348.255\frac{gm}{s^2} = 0.348255\frac{kgm}{s^2} = 0.348255N$$

Contact Length (L)

L = length of contact the tube has with the bottom platen.

Assumption: Length of contact with the PDMS is equal to the length of contact of the aluminum funnel. L = 2mm = 0.002m

Therefore, the compressive strength (CD) can be calculated below.

$$CD = \frac{F}{A} = \frac{0.348255\frac{kgm}{s^2}}{2(0.295mm)(2mm)} = 0.2951\frac{\frac{kgm}{s^2}}{mm^2} = 0.2951\frac{N}{mm^2} = 295.13kPa$$

APPENDIX C
ZEBRAFISH SOLUTION CONCENTRATION

All solution concentrations (Cisplatin, DASPEI, MS-222, Dissolution products) were created using Embryo Media as the base.

Cisplatin: (50µM – 1mM)

$$Cisplatin\ (MW) = \frac{300.05g}{mol}$$

$$1M = \frac{1mol}{1000ml} \Rightarrow \frac{300.05g\ Cisplatin}{mol} \cdot \frac{1mol}{1000ml} \Rightarrow \frac{0.30005g\ Cisplatin}{1ml\ media}$$

$$\underline{For\ 50\mu M = 0.000050M}$$

$$0.000050M \times \frac{0.30005g\ Cisplatin}{1ml\ media} = \frac{0.000015g\ Cisplatin}{ml\ media}$$

$$with\ 10ml\ of\ soltuion$$

$$10ml \times \frac{0.000015g\ Cisplatin}{ml\ media} = \underline{\underline{0.00015g\ Cisplatin\ in\ 10ml\ of\ media}}$$

$$\underline{For\ 1mM}$$

$$0.001M \times \frac{0.30005g\ Cisplatin}{1ml\ media} \times 10ml\ solution \Rightarrow \underline{\underline{0.003g\ Cisplatin\ in\ 10ml\ media}}$$

DASPEI: (1mM)

$$DASPEI\ (MW) = \frac{380.27g}{mol}$$

$$1mM = 0.001\frac{mol}{1000ml}$$

$$\frac{380.27g\ DASPEI}{mol} \times \frac{0.001\ mol}{1000ml} = \frac{0.00038027g\ DASPEI}{ml\ media}$$

$$\underline{Want\ 10ml\ of\ solution}$$

$$10ml \times \frac{0.00038027g\ DASPEI}{ml} \Rightarrow \underline{\underline{0.0038027g\ DASPEI\ in\ 10ml\ media}}$$

MS-222: (10µg/ml-250mg/L)

$\underline{\textit{To make } 10\mu g/ml \textit{ solution}}$:

$$10ml\ solution: 10ml \times \frac{10\mu g}{ml} = \underline{\underline{100\mu g\ in\ 10ml\ media\ (0.0001g)}}$$

$\underline{\textit{For Euthanisa}}$:

$$\frac{250mg\ MS-222}{L} \times \frac{L}{1000ml} = \frac{0.25mg\ MS-222}{ml} \times 10ml\ media \Rightarrow \underline{\underline{2.5mg\ MS-222\ in\ 10ml\ media}}$$

Buffered with

$$\frac{\sim 200mg\ NaHCO_3}{L} \times \frac{L}{1000ml} = \frac{0.2mg\ NaHCO_3}{ml} \times 10ml\ media \Rightarrow \underline{\underline{2mg\ NaHCO_3\ in\ 10ml\ media}}$$

Dissolution Products: (0.5M-0.1M)

Embryo Media contains 0.035g $NaHCO_3$ /ml. In a 100ml of embryo medial there is 0.00035g $NaHCO_3$ per ml of embryo media (or a total of 0.035g). A 0.5M $NaHCO_3$ solution contains 4.2g of $NaHCO_3$ per 100ml of solution.

$$\frac{0.5mol\ NaHCO_3}{L} \times \frac{84.01g\ NaHCO_3}{mol} \times \frac{L}{1000ml} = \frac{0.042g\ NaHCO_3}{ml} \times 100ml \Rightarrow 4.2g\ NaHCO_3$$

Therefore, to make 0.5M solution using embryo media as the base instead of DI water subtract 4.2g-0.035g to get 4.1659g $NaHCO_3$ in 100ml. We only need 10 ml of solution so we divided by 10 resulting in 0.4165g $NaHCO_3$ in 10 ml of solution. 0.25M and 0.1M solutions were created by dividing 0.4165 by 2 (0.208g) and 5 (0.083g in 10ml of embryo media) respectively.

The molar concentration of $NaHCO_3$ in embryo media was determined using the molecular weight of $NaHCO_3$ (84.01g/mol) and the following formula (given the amount of $NaHCO_3$ in embryo media is 0.00035g/ml). Therefore, the molar concentration of $NaHCO_3$ used to dissolve the tube (0.1M) was more then 20 times greater then the molar concentration in embryo media.

$$\frac{0.00035g}{ml} \times 1000ml = \frac{0.35g}{L} \times \frac{1mol}{84.01g} = 0.00416\frac{mol}{L}(M)$$

APPENDIX D

NEUROMAST FLUROESCENT INTENSITY

Neuromast P4 was isolated from each Zebrafish treatment and the image colors were inverted in order to clearly see the individual hair cells. The results (Table D-1) show that the fluorescent intensity (dark purple area) (Figure D-1) was significantly higher for the hair cells exposed to the dissolution products and negative no-treatment control when compared to the positive Cisplatin control. It was also shown that there are more viable hair cells per neuromast and a larger effective neuromast area in those Zebrafish exposed to the dissolution products and negative no-treatment control than the positive Cisplatin control.

Table D-1: Fluorescent intensity, neuromast area, and hair cell number based on Zebrafish treatment.

	No Treatment Negative Control			Positive Control (Cisplatin)			Dissolution Products		
	Fluorescence	Area	#	Fluorescence	Area	#	Fluorescence	Area	#
1	218.19	299.4	7	106.25	185.0	6	190.12	214.2	6
2	205.74	252.6	8	160.37	244.0	6	161.79	111.4	4
3	197.37	288.3	8	81.75	131.1	3	135.49	110.9	4
4	149.02	219.3	7	151.14	313.0	6	194.39	214.2	3
5	199.3	215.8	6	199.52	117.5	4	73.92	224.8	8
6	180.48	225.8	8	171.83	173.9	5	183.32	174.9	5
7	141.89	376.6	6	145.69	134.6	4	105.82	441.1	6
8	150.61	302.0	6	157.88	112.9	4	134	265.7	6
9	195.03	328.7	7	128.3	128.0	4	229.79	240.5	5
10	88.14	705.7	4	109.96	184.5	3	134	501.6	7
11	182.47	382.6	9	108.08	178.5	4	127.27	200.6	4
12	224.8	218.8	8	166.89	250.5	7	174.41	455.7	9
13	166.09	499.6	9	127.79	156.8	5	189.14	335.2	7
14	139.13	320.1	5	81.35	226.8	5	161.51	373.5	7
15	124.26	321.1	6	112.04	159.3	4	171.93	337.7	7
16	--	--	--	125.15	179.0	6	155.05	317.1	7
17	--	--	--	128.11	163.8	4	152.65	113.9	2
18	--	--	--	124.41	217.8	5	165.86	368.0	8
Average	170.8	330.4	6.9	132.6	180.9	4.7	157.8	277.8	5.8
St. Dev.	38.2	129.0	1.4	31.4	52.7	1.1	36.1	120.2	1.9

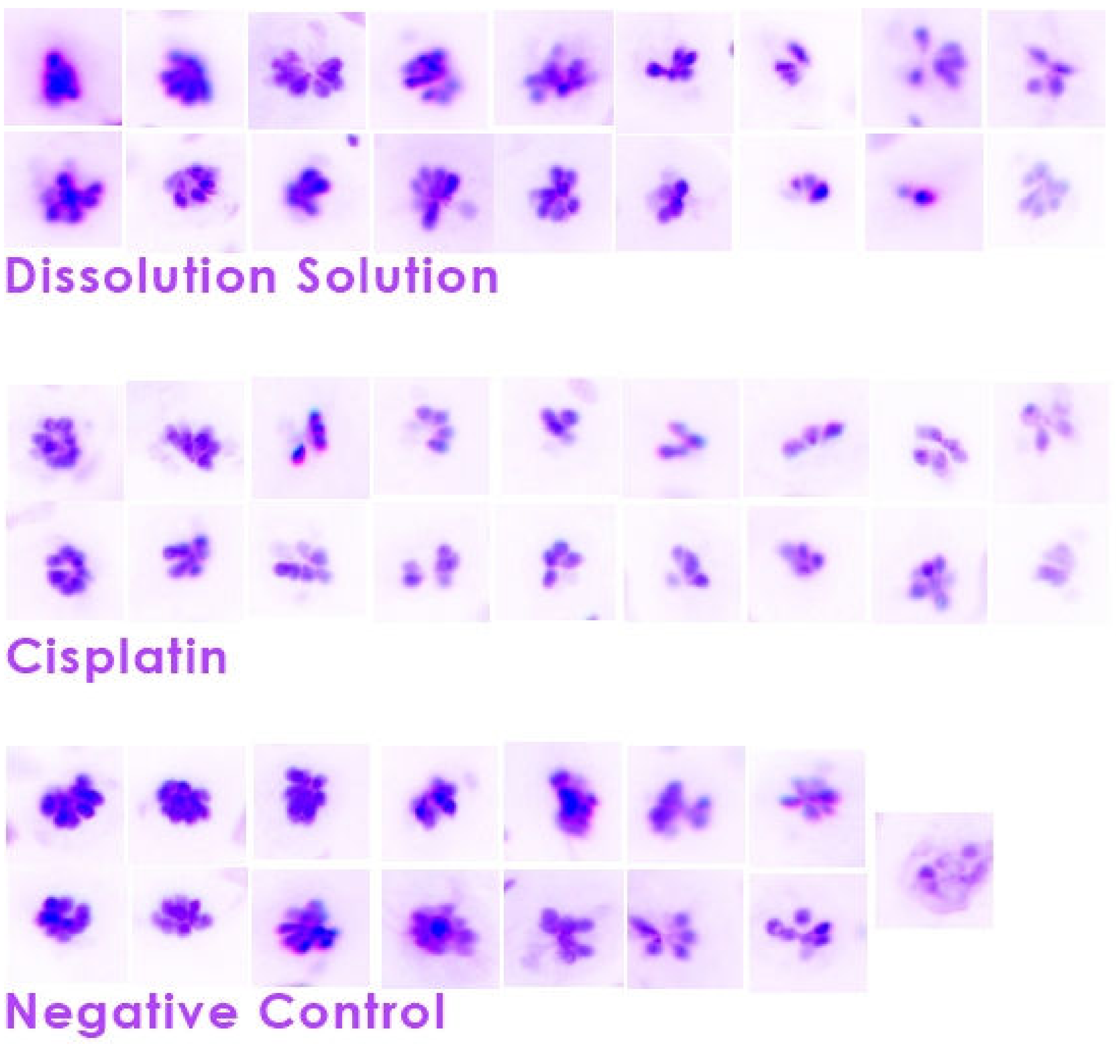


Figure D-1: Zebrafish neuromast P4 hair cells for each experimental treatment.

APPENDIX E
DISSOLUTION SOLUTION QUANTITY

The amount of each dissolution component in solution was calculated for calcium alginate, NaCl, and $NaHCO_3$.

Calcium Alginate:

Molecular Weight = $\frac{121g\ Ca\ Alginate}{mol}$

Molar Con. (Tube weight = 0.011g Ca Alginate) =

$$0.011g\ Ca\ Alginate \times \frac{mol}{121g\ Ca\ Alginate} = 9.09 \times 10^{-5}\ mol$$

NaCl:

Molecular Weight = $\frac{28g\ NaCl}{mol}$

Molar Con. (at 0.85%) (0.5ml solution)=

$$\frac{8.5g\ NaCl}{L} \times \frac{mol}{28g\ NaCl} = 0.303 \frac{mol}{1000ml} \times 0.5ml = 1.515 \times 10^{-4}\ mol$$

$NaHCO_3$:

Molar Con. [0.1M] (0.5ml solution) = $0.5ml \times \frac{0.1\ mol}{1000ml} = 5 \times 10^{-5}\ mol$

Molar Con. [0.5M] (0.5ml solution) = $0.5ml \times \frac{0.5\ mol}{1000ml} = 2.5 \times 10^{-4}\ mol$

Molar Con. [1.0M] (0.5ml solution) = $0.5ml \times \frac{1.0\ mol}{1000ml} = 5 \times 10^{-4}\ mol$

Molar Con. [2.0M] (0.5ml solution) = $0.5ml \times \frac{2.0\ mol}{1000ml} = 1 \times 10^{-3}\ mol$

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BIOGRAPHICAL SKETCH

The author was born in 1978, in Manhattan, New York to an elementary school teacher and a secretary. He has one younger sibling, a very talented actress and artist, Nicole. In 1980, the author's parents moved to West Palm Beach, Florida. It was here that the author grew up and discovered the wonderful world of engineering. As a child, he constantly took apart everything he could get his hands on and used the parts to create his own inventions. It was here that he fell in love with science, a lifelong passion that is with him constantly. In May 2002, he received his Bachelor of Science in engineering science with a minor in biomechanics from the University of Florida and entered graduate school in the Mechanical Engineering Department. Shortly before finishing his bachelor's degree, he started working for Dr. Roger Tran-Son-Tay in the Biorheology Lab and he continues pursuing his research there today. In August 2005, he received his Masters in Engineering in mechanical engineering from the University of Florida. In the summer of 2001, he met the love of his life Amy and from then on knew that he had found his soul mate. On February 1, 2004, he asked her to marry him, and on June 6, 2006, they were married. He and his wife currently live in Gainesville, Florida.