Progress Toward Improved Drug Delivery to the Inner Ear: Methods of Measuring Intracochlear Drug Distribution and Materials-Based Approaches for Controlled Release

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Progress Toward Improved Drug Delivery to the Inner Ear: Methods of Measuring Intracochlear Drug Distribution and Materials-Based Approaches for Controlled Release

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements

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Hearing loss is the most common sensory deficit and has a profound impact on quality of life. Despite the emergence of promising therapeutic candidates for hearing restoration, the dearth of safe and efficacious methods of delivering drugs to the isolated cochlea and its sensorineural epithelium represents a potential barrier to clinical translation of investigational therapies. To address this, novel drug delivery approaches that aim to improve the spatiotemporal distribution of drug substances along the cochlear axis are being developed and evaluated in preclinical animal models. However, the small volume and anatomic isolation of the cochlea make pharmacokinetic assessment difficult, and existing techniques of measuring drug distribution in the cochlea have limitations. We have developed an approach to measuring drug distribution directly in the cochlea’s sensory epithelium, utilizing targeted fluorescent drug-surrogate substances that can be quantified along the cochlear axis in whole-mount preparations with excellent spatial resolution. We evaluate several fluorescent substances as candidate compounds, which are delivered intracochlearly \textit{in vivo} by a precise microfluidic injection prior to cochlear dissection, and we compare the distributions with a computational model of drug distribution within the scala tympani compartment. We also measure the distribution of the lead candidate, FM 1-43 FX at multiple timepoints after injection, up to 72 hours. Finally, we propose novel materials-based strategies for injectable polymer vehicles that could be applied as controlled release vehicles for intracochlear drug delivery applications.
Acknowledgements

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1 Thesis Introduction

Sensorineural hearing loss (SNHL) occurs from damage to the cochlea’s sensorineural epithelium and represents the most common sensory deficit. Although not considered a life-threatening condition, SNHL has significant negative impacts on the quality of life for those affected and, unfortunately, limited treatment options currently exist. Recent discoveries of the pathophysiology of various forms of hearing loss and the identification of molecular targets for therapeutic intervention promise to turn the page on what was previously considered an irreversible deficit. However, among the many challenges that emerging drug candidates face in clinical trials, one of the most significant barriers is the ability to reach their intended targets inside the tiny and anatomically isolated fluid labyrinth that is the inner ear. The effectiveness of new drugs will centrally rely on safe and efficacious methods of delivering drugs to the cochlea’s sensorineural epithelium, which transduces acoustic stimuli into afferent auditory neural signals. To date a clinically viable method of controlled drug delivery to the inner ear has not emerged, because proposed approaches suffer at least one of the following limitations:

i. Delivered substances do not reach the sensorineural targets in effective concentrations;

ii. Delivered substances are not distributed well throughout the cochlea;

iii. Delivered substances are not maintained within the therapeutic window for long enough;

or

iv. Delivery approach is too invasive, and the perceived risk of damage to the cochlea outweighs the potential benefit.
Therefore, the research and development of novel delivery strategies continues. Prior to testing in humans, the safety and efficacy of newly proposed drug delivery methods must be first evaluated in animal models. Of the several species commonly used for auditory research, the guinea pig has been the most widely used model for the study of drug delivery to the inner ear. These experiments typically entail a surgical intervention to administer a drug or drug delivery device, a controlled-release modality in the form of a reservoir, and a method of measuring the concentration of the substance delivered in the cochlea. Several methods of inferring spatiotemporal drug distribution within the cochlea have been reported, yet a robust and straightforward experimental approach to evaluating pharmacokinetics in the cochlea is lacking. The limitations of existing pharmacokinetic evaluation techniques include one or more of the following:

i. The method of evaluation is significantly impacted by experimental artifacts;

ii. The method of evaluation does not directly measure intracochlear concentration;

iii. The method of evaluation lacks spatial resolution along the cochlear axis; or

iv. The concentration of drug is not directly measured in the target sensorineural cells.

Ultimately, the emergence of effective therapeutics to prevent and/or reverse sensorineural hearing loss in humans will require the development of effective delivery approaches, which in turn, relies on the establishment of robust pharmacokinetic evaluation methods. These unsolved issues will only become more urgent as more investigational therapies are advanced to the clinical stage. The overall goal of this thesis is to investigate new approaches for intracochlear pharmacokinetic evaluation and drug delivery.
1.1 Significance of this thesis

The largely unmet clinical need of viable and effective drug delivery strategies is widely recognized and required for the translation of many emerging drugs to treat hearing loss. Though perhaps less readily apparent, but of fundamentally equal importance, is the establishment of methods to quantify drug delivery to the inner ear, which is required for the development of novel inner ear drug delivery technologies. This thesis presents several innovative approaches to address these issues, including the first comprehensive study of fluorescent drug-surrogate tracers quantified in the sensorineural epithelium after intracochlear delivery. The successful demonstration of a straightforward method to measure drug distribution with high spatial resolution along the cochlear axis, presented here, will serve as an important tool for evaluating new intracochlear drug delivery approaches in the future. Lastly, I discuss a novel materials engineering approach to tune the mechanical properties of injectable gels that could be developed as drug delivery reservoirs for drugs applied to the middle and inner ear.

1.2 Specific Aims

The overall objectives of this thesis are to develop new tools to evaluate intracochlear drug delivery and investigate novel materials-based platforms that may be used as controlled release reservoirs for local drug delivery. The focus of this work involves the study of drug distribution along the cochlear axis, both in the scala tympani compartment of the cochlea and the sensorineural cells that are the therapeutic targets of most emerging therapeutics.

The specific aims of this thesis that address these objectives are as follows:

i. Construct and implement a computational model for spatiotemporal drug distribution in the cochlea’s scala tympani compartment.
ii. Identify and investigate intracochlearly-delivered fluorescent substances with affinity for the cochlea’s sensorineural cells in vivo, to serve as lead candidate tracers in extended studies.

iii. Develop a method of quantifying the distribution of drug concentration along the sensory epithelium after intracochlear delivery and provide an analysis of the measured distribution with computational models of drug transport in the cochlea.

iv. Study the fluorescence distribution after intracochlear delivery of a leading fluorescent substance candidate at multiple timepoints to provide an additional perspective on cochlear pharmacokinetics and drug transport.

v. Synthesis and characterization of hydrogel materials with tunable mechanical properties for potential applications in local therapy of the middle and inner ear.

1.3 Thesis Outline

This thesis is presented in seven chapters. This chapter introduces the thesis and its specific aims. Chapter 2 provides the contextual background information and sets up a framework of the challenges and opportunities relating to therapy of the inner ear. This chapter provides a detailed literature review that encompasses the structure and function of the cochlea as the peripheral sensory organ of hearing, the pathophysiology of hearing loss, and emerging therapeutic approaches to address the unmet clinical need. Further, it reviews the approaches and technologies that have already been developed to deliver drugs locally to the inner ear. Special attention is paid to methods of intracochlear drug delivery that overcome the systemic and local barriers to delivering drugs to the cochlea.

Chapters 3-6 cover the original research work conducted in this thesis. Chapter 3 describes a computational method to predict drug distribution in the scala tympani using parameters derived
from the body of experimental pharmacokinetic literature. Chapters 4 and 5 describe the establishment of a fluorescence-based imaging approach to measuring and quantifying drug distribution in the cochlea’s sensory epithelium in the paradigm of a single intracochlear injection. Chapter 6 introduces the design and characterization of polymer-based material systems that may be implemented in the future as controlled-release vehicles for cochlear therapy.
2 Background

Sections of this chapter were adapted from a review article published by AM Ayoob and JT Borenstein [1].

2.1 Epidemiology and socioeconomic burden of hearing impairment

Drug delivery to the inner ear has been an area of increasing interest, as investigators seek improved methods to administer therapeutic agents for the treatment of auditory and vestibular system disorders, including sensorineural hearing loss (SNHL), tinnitus, and vertigo. More than 250 million people worldwide suffer disabling hearing loss [2]. The most common type of sensory impairment in humans is SNHL, currently affecting over 300 million people worldwide, with a projected 65 million Americans affected by 2030 [3]. More than 25 million people have been affected by tinnitus, which is linked to SNHL, and 2-3 of every 1,000 children are born with significant SNHL [4]. Another 45,500 individuals are diagnosed with Meniere’s Disease (MD) each year, a syndrome which impacts both hearing and balance and results in SNHL due to damage to the auditory system [4]. In the US military, noise induced hearing loss (NIHL) and tinnitus represent two of the most common disabilities, with tinnitus alone expected to cost the government over $2.2 billion in veteran disability compensation during 2014 [5]. According to a 2012 study conducted by AARP Services, hearing impairment in senior citizens affects quality of life to a greater degree than hypertension, stroke, osteoporosis, sciatica, and cancer [6]. For the clear majority of cases, hearing loss can be attributed to disorders of, or damage to the peripheral auditory system, including the cochlear hair cells and the auditory nerve.
2.2 The cochlea is the peripheral sensory organ of hearing

The inner ear comprises the fluid-filled organs of both hearing and balance. The cochlea’s sensory epithelium, which transduces sound pressure waves into neural signals, is 32 mm long in humans and is coiled structure housing three parallel fluid-filled cavities called scalae. The scala tympani (ST) contains perilymph and terminates at the RWM, an entryway for diffusion of drug substances from the middle ear, as well as a potential location for cannula insertion, which will be discussed later. Perilymph contains a high sodium concentration, and is similar in composition to the CSF, but exhibits a significantly higher protein concentration[8]. The scala vestibuli (SV) also contains perilymph and terminates at the oval window membrane and stapes footplate, where mechanical motions from the middle ear are transmitted to the inner ear fluids. The ST and the SV are continuous with each other via the helicotrema at the cochlear apex. The scala media (SM) contains endolymph that bathes the apical surface of the inner and outer hair cells. The endolymph is a
unique extracellular fluid, in that it contains a high potassium concentration. The so-called “endocochlear potential” describes the steep electrochemical gradient (+150 mV) between endolymph and perilymph, which functions as a battery powering mechanoelectrical transduction. The vestibular system, which detects linear and rotational accelerations, consists of the utricle, the saccule, and the three semicircular canals. These vestibular compartments contain endolymph, which is continuous with the SM of the cochlea.

The cochlear sensory epithelium consists of one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) mounted on the basilar membrane, and coils parallel with the scalae, dividing the ST and the SM. Sound pressure waves enter the external ear canal, vibrating the tympanic membrane and the ossicular chain, which comprises the malleus, incus, and stapes. The stapes behaves as a piston that gives rise to pressure waves in the cochlea, setting the basilar membrane into motion. IHCs respond to local displacements of the basilar membrane by releasing neurotransmitters to activate auditory nerve fibers. Loss of function of either the hair cells or the auditory neurons results in hearing loss.
2.3 Pathophysiology and forms of SNHL

SNHL frequently results from damage to the inner hair cells (IHC)s, outer hair cells (OHC)s, or the afferent spiral ganglion neurons (SGN)s that innervate the sensory hair cells. Insults that cause damage to these structures include acute acoustic trauma, long-term noise exposure, aging, aminoglycoside drugs, chemotherapeutics, Meniere’s syndrome, viral infection, meningitis, autoimmune disease, and genetic abnormalities. Currently, most hearing-impaired patients seeking treatment receive either hearing aids or surgically implanted cochlear prostheses. However, novel therapeutic strategies, summarized in Table 2.1, are currently being investigated to prevent or reverse SNHL, and to treat a range of other auditory diseases.
### Table 2.1 Therapeutic Strategies for the treatment of HL by type

<table>
<thead>
<tr>
<th>Differential Diagnosis</th>
<th>Therapeutic Strategy</th>
<th>Mechanism</th>
<th>Examples</th>
</tr>
</thead>
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<tr>
<td><strong>Syndromic Deafness</strong></td>
<td>Gene Therapy</td>
<td>Gene modulation/replacement</td>
<td>Myosin VIIA replacement</td>
</tr>
<tr>
<td></td>
<td>Potassium Homeostasis</td>
<td>Ion channel modulation</td>
<td>Ezogabine</td>
</tr>
<tr>
<td><strong>Traumatic-brain-injury-associated Hearing Loss</strong></td>
<td>Antioxidants</td>
<td>Scavenge ROS</td>
<td>Glutathione, D-methionine</td>
</tr>
<tr>
<td><strong>Acoustic Overexposure</strong></td>
<td>Corticosteroids</td>
<td>Unclear</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td></td>
<td>Apoptosis Inhibition</td>
<td>Mitogen Activated Kinase Pathway</td>
<td>Peptide Inhibitor</td>
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<tr>
<td></td>
<td>Antioxidants</td>
<td>Scavenge ROS</td>
<td>Glutathione, D-methionine</td>
</tr>
<tr>
<td><strong>Age-related HL</strong></td>
<td>Antioxidants</td>
<td>Scavenge free radicals</td>
<td>Glutathione, D-methionine</td>
</tr>
<tr>
<td></td>
<td>Synapse Maintenance</td>
<td>Neurotrophic support</td>
<td>Neurotrophin-3</td>
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<tr>
<td></td>
<td>Hair Cell Regeneration</td>
<td>Notch-inhibition</td>
<td>γ-secretase inhibitor</td>
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<td></td>
<td>Potassium Homeostasis</td>
<td>Ion channel modulation</td>
<td>Ezogabine</td>
</tr>
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<td><strong>Chemotherapy-induced HL</strong></td>
<td>Chelating agents</td>
<td>Platinum chelation</td>
<td>Sodium thiosulfate</td>
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<td></td>
<td>Apoptosis Inhibition</td>
<td>Caspase Inhibition</td>
<td>Caspase-3,9 inhibitors</td>
</tr>
<tr>
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<td>Develop non-ototoxic aminoglycosides</td>
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<td>N/A</td>
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<td><strong>Meniere’s Disease HL</strong></td>
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<td><strong>Idiopathic SSNHL</strong></td>
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<td>Unknown</td>
<td>Dexamethasone</td>
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<tr>
<td></td>
<td>Antivirals</td>
<td>Viral DNA polymerase inhibition</td>
<td>Acyclovir</td>
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<td></td>
<td>Anticoagulants</td>
<td>Defibrinogenation</td>
<td>Viprinex</td>
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<td>Corticosteroids</td>
<td>General immunosuppression</td>
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<tr>
<td></td>
<td>Immunosuppression</td>
<td>TNF inhibition</td>
<td>Etanercept</td>
</tr>
</tbody>
</table>

It has been known for decades that acute noise overexposure damages the cochlear sensory epithelium, particularly the outer hair cells and their stereocilia bundles, and results in permanent auditory threshold elevations [11, 12]. This type of hearing loss is referred to as noise induced hearing loss (NIHL). Multiple strategies for reversing the effects of acute noise induced hearing loss...
loss are being developed, including regeneration of the auditory hair cells from supporting cells, and [13, 14], as well as apoptosis inhibitors that prevent the death of hair cells if administered immediately after the insult [15]. Recently, a more insidious form of hearing loss, termed “hidden hearing loss”, has been recently discovered that occurs from gradual “pruning” of the synapses between the auditory neurons and the cochlear inner hair cells [16]. This type of hearing loss occurs from repeated exposure to noise, even at levels that are not lethal to the hair cells [17]. This type of hearing loss requires a strategy that promotes neurite outgrowth and/or maintains the hair cell synapses; NT-3, a neurotrophic factor, has been identified as a promising therapeutic agent that maintains the synapses and can even stimulate the formation of new synapses [18-20].

Systemic exposure to ototoxic drugs is a major risk factor for cochlear hair cell death and the development of SNHL. Multiple rounds of platinum-based chemotherapies for the treatment of solid tumors have been shown to cause hearing loss in a relatively large portion of the patient populations [21]. Countermeasures such as systemic dosing with platinum binders have shown moderate efficacy in reducing auditory damage but may also reduce the desired therapeutic effect of chemotherapy [22], and are therefore sometimes contraindicated. This situation represents an ideal opportunity for local intervention, which may reduce the local toxic effect in the ear without hindering the desired systemic effect of the chemotherapeutic drug. Similarly, aminoglycoside antibiotics are highly toxic to the outer hair cells in the high frequency regions of the cochlea [23], and yet must be administered in some cases to fight systemic infections [24]. In one study, over 77% of patients demonstrated decreased cochlear function after 56 days of streptomycin treatment [25].

Meniere’s Disease (MD), autoimmune inner ear disease (AIED), and sudden sensorineural hearing loss (SSNHL) are less common indications, but all typically result in profound hearing loss. While
the pathophysiology of MD remains unknown and may encompass several different etiologies, most cases of MD terminate in profound deafness in either one or both ears[26]. High dose steroids may ameliorate some of the hearing loss caused by MD[27], but long-term dosing is precluded by the unfavorable side effects of systemic steroid treatment. Likewise, high doses of steroids are highly effective in staving off AIED, but the associated side effects eventually become unbearable in many cases[28]. Short course steroids have been highly effective in approximately 61% of SSNHL patients[29], and SSNHL may, therefore, represent an indication where on-demand short course dosing is appropriate. **Figure 2.3** summarizes the forms of SNHL by their differential diagnosis.

---

**Figure 2.3 Differential Diagnoses of Sensorineural Hearing Loss**
Tinnitus, the perception of sound without an external stimulus, occurs in about 10-15% of the population and severely impairs the quality of life in 1-2% of all people[30]. Unfortunately, the etiology of tinnitus is controversial, with potential involvement of both peripheral and central neural components[31]. It is well known that hearing loss is sometimes associated with tinnitus[32], and immediate treatment with steroid medications following sudden hearing loss may be effective in preventing the development of tinnitus in some cases[33]. However, for many patients, tinnitus is a persistent problem that may or may not be associated with a sudden hearing loss.

2.4 Approaches to drug delivery for inner ear therapy

Advances in understanding the physiological and biomolecular basis of inner ear diseases have accelerated the search for novel therapeutics, including apoptosis inhibitors, cytokines, neurotrophic factors, antioxidants, gene vectors, siRNA, and cell-based therapies[34]. However, despite the increasing number of candidate compounds, the inner ear remains among the most challenging organs for targeted drug delivery. Local entry of systemically circulating drugs is impeded by the blood-cochlea barrier, which blocks entry of many small molecules and proteins dissolved in the plasma[35]. Further, systemic dosing is associated with deleterious side effects that often preclude prolonged therapy, such as those seen in long-term corticosteroid regimens for management of AIED [36]. In response to these challenges, intratympanic (IT) injection of a drug solution into the middle ear, aimed at increasing local absorption at the inner ear and avoiding systemic side effects, has become the preferred route for many clinical applications, and is commonly used for treatment of intractable Meniere’s Disease [37]. It is thought that the major site of drug absorption between the middle and inner ear is the Round Window Membrane (RWM),
which is in the medial wall of the middle ear in the base of the cochlea. However, there are significant anatomical limitations associated with IT delivery, including rapid clearance of drug solution from the middle ear via the Eustachian tube, and patient to patient variability in both the size and permeability of the RWM [38]. These impediments to local delivery are compounded by the remote location and relative small size of the inner ear. The cochlea and the vestibular system are housed within the petrous bone, the hardest bone in the body, and direct surgical access is blocked by the adjacent mastoid bone. The total volume of the inner fluids in humans is approximately 200 μL, and that volume in the typical preclinical animal models, such as guinea pigs, is generally smaller by about an order of magnitude [39].

To circumvent challenges faced by traditional methods for inner ear drug delivery, including oral administration, intravenous infusion[40], intramuscular injection[41], and IT injection, several groups have developed direct intracochlear delivery systems suitable for surgical implantation[42]. Initial attempts included osmotic pumps[43], which have appropriate dimensions for implantation, but have limited delivery duration and lack control over delivery kinetics. Recently, active microfluidic systems have emerged as means to precisely meter drug concentration and promise both extended and on-demand drug delivery to the cochlea, either as a standalone implant or in conjunction with a cochlear implant[44]. Table 2.2 summarizes approaches for intracochlear delivery.
**Table 2.2 Approaches to drug delivery for inner ear therapy**

<table>
<thead>
<tr>
<th>Delivery Approach</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Drugs tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic Delivery</td>
<td>Minimally invasive</td>
<td>Low blood perfusion of inner ear/blood cochlea barrier</td>
<td>Dexamethasone/Streptomycin</td>
</tr>
<tr>
<td>Intratympanic Injection</td>
<td>Moderately invasive, considered safe</td>
<td>Drainage through eustachian tube/limited RWM permeability</td>
<td>Dexamethasone/Gentamicin</td>
</tr>
<tr>
<td>RWM MicroWick™</td>
<td>Semi continuous delivery</td>
<td>Limited RWM permeability</td>
<td>Dexamethasone/Gentamicin</td>
</tr>
<tr>
<td>RWM Catheter</td>
<td>Continuous delivery</td>
<td>Surgically invasive/limited RWM permeability</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>Cochlear Prosthesis-Mediated Delivery</td>
<td>Direct access to sensorineural cells</td>
<td>Highly invasive, only indicated for adjunct to cochlear implantation</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>Intracochlear Delivery</td>
<td>Direct access to sensorineural cells</td>
<td>Surgically invasive, requires violating inner ear barriers</td>
<td>Gene vectors</td>
</tr>
</tbody>
</table>

### 2.4.1 Systemic delivery

Systemic methods of drug delivery, such as oral delivery, rely on the cochlear blood supply to facilitate transport of drug molecules to the inner ear structures. However, diffusion of substances from the systemic circulation into the inner ear fluids is severely hindered by the blood-cochlea barrier (BCB). Similar to the blood-brain barrier (BBB), the BCB is set up by tight junctions between endothelial cells of the inner ear vasculature that prevent diffusion of many small molecules and proteins [35]. The cochlear blood supply emanates from the anterior inferior cerebellar artery, and total cochlear blood flow in humans is estimated to represent less than one one-millionth of total cardiac output [45]. Furthermore, several otologic disorders are suspected to be associated with reduced cochlear blood flow and ischemia including NIHL and endolymphatic
hydrops[46]. The vascular areas of the cochlea are mostly limited to the modiolus, the organ of Corti, and the stria vascularis. The stria vascularis, a highly vascularized structure, is thought to be the major site associated with selective transport of certain solutes into the endolymph [47].

![Figure 2.4 Drawing of cochlear vasculature supply [48]](image)

**Figure 2.4 Drawing of cochlear vasculature supply [48]**

### 2.4.2 Delivery by absorption through the round window membrane

Intratympanic delivery is the current clinical standard for local drug delivery to the inner ear. In this procedure, a concentrated drug solution is injected through the tympanic membrane in an outpatient setting. Drugs administered to the middle ear diffuse into the cochlea primarily via the round window membrane (Figure 2.5), but also enter through the oval window membrane and the cochlear bone.[49] Dexamethasone for the treatment of sudden idiopathic hearing loss and gentamicin for intractable vertigo associated with Meniere’s disease are the two most common substances applied by the intratympanic route.[50, 51] However, there are significant limitations of intratympanic delivery that challenge its clinical utility, especially for substances that do not readily permeate the round window membrane. The first limitation is achievable duration of
therapy. This is because drug solutions only remain in contact with the round window membrane for approximately 1 hour, while the patient is maintained in the prone position with the treated ear facing upward, and readily drain through the Eustachian tube after the treatment period. The second limitation is the limited permeability of the round window membrane, which limits the flux of drug through the membrane and is poorly permeant to charged substances and macromolecules, such as protein therapeutics[52, 53]. This problem is exacerbated by the variable thickness of the human round window membrane, the occurrence of “false” round window membrane folds, and the increased thickness of the round window in pathological cochleae [38]. Finally, delivery through the round window membrane is associated with substantial concentration gradients from base to apex, owing to the diffusional limitation of drugs entering the base of the scala tympani compartment coupled with the relatively fast rates of radial clearance and cochlear aqueduct homeostasis [54, 55].

Figure 2.5 Schematic of intratympanic injection [56]
To address the important problem of Eustachian tube drainage, several strategies have been developed to maintain drug levels at the round window membrane in an effort to prolong contact time. Some of these strategies are described below:

Rubel and colleagues placed Ethylenvinylacetate (Elvax®) disks on the RWM to induce prolonged auditory nerve blockade in animal models, and to investigate morphological changes on cochlear nucleus neurons[57]. More recently, controlled release polymer depots have been investigated for therapeutic purposes in both preclinical and clinical settings. In preclinical models, BDNF delivery in a slow release gelatin formulation was significantly more effective in reducing secondary degeneration of spiral ganglion neurons compared to a single injection of a BDNF solution[58]. Gelfoam® loaded with gentamicin was placed at the RWM via tympanostomy in 32 patients with MD, achieving control of vertigo in 75% of patients and with hearing preserved in 90% of patients[51]. Poloxamers are a family of temperature-sensitive triblock copolymers composed of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) domains. For instance, OTO-104 is poloxamer formulation containing micronized dexamethasone, which can be injected through a small gauge medical needle at low temperatures [59]. Upon intratympanic injection of the liquid polymer solution into the RWM niche, OTO-104 rapidly undergoes a thermodynamically induced phase change, called the sol-gel transition, forming a solid hydrogel drug depot in contact with the RWM. Gelation at physiological temperatures is mediated by physical crosslinking, resulting from hydrophobic interactions between the PPO domains [60]. OTO-104 is currently in phase 2 trials for treatment of MD[61], and may also see
use for treatment of AIED. The effects of the amount of relative poloxamer and drug loading on
the release rates has been studied and optimized in vitro[62].

The Silverstein MicroWick® is a porous, polyvinyl acetate cylinder, 1mm in diameter and 9 mm
in length, that transverses the TM via a tympanostomy tube [63]. It straddles the TM at one end
and contacts the RWM at the other. Drugs applied to the external ear canal travel through the
MicroWick™ by means of capillary action and are presented at the surface of the RWM for
absorption into the inner ear. The Microwick is primarily used for gradual titration of gentamicin
in some MD patients[64, 65], but has also been used to dose corticosteroids to treat SSNHL[66,
67]. Disadvantages of this approach include persistent tympanic perforation after removal, middle
ear infection, and cholesteatoma[68].

IntraEar® Round Window μ-Cath™ is a multi-lumen microcatheter system designed to be used
with an external pump to provide continuous perfusion of the RWM. The catheter tip is placed in
the RWM niche during a procedure that involves drilling a tunnel through the temporal bone,
inferior to the TM. The catheter is inserted into the middle ear through the surgical passage and
remains in position at the RWM niche for several weeks, until it is removed[69]. Another version
of this device, called the Round Window E-Cath™, also contains an electrode in the tip for
continuous monitoring of electrical activity in the cochlea during treatment. Modeling studies have
shown that continuous delivery to the RWM may reduce the base-apex concentration gradient and
increase the amount of drug that reaches the apical portions of the cochlea, including the frequency
locations that encode speech information[70]. This system has been used in patients for continuous
application of gentamicin[71] and corticosteroids[72] for treatment of MD and SSNHL,
respectively. Although complications have been associated with this semi-implantable device,
including catheter dislocation, catheter obstruction, formation of granulation tissue in the middle
ear, and persistent TM perforation following removal, the device is considered to be generally safe for use in humans [69].

Within the past decade, nanotechnology-based drug delivery systems including silica nanoparticles[73], PLGA nanoparticles[74], lipid nanocapsules[75], liposomes[76], hyperbranched polylysine nanoparticles[77], and magnetic nanoparticles[78] have received increased attention as potential inner ear drug delivery vehicles [79]. Some of these formulations aim to achieve well-controlled delivery and improved RWM transport characteristics for gene vectors, protein-based therapies, and poorly water-soluble small molecule drugs. Youm and colleagues demonstrated that PEGylated PLGA nanoparticles are internalized in HEI-OC1 cochlear cells via a clathrin-dependent pathway; suggesting that these particles could serve as nano-sized drug reservoirs localized within the hair cells[80]. Zou and colleagues utilized a tyrosine receptor kinase (TrkB) ligand conjugated to the exterior surface of liposomes, to target and anchor the nanoparticle to the spiral ganglion neurons; early gene therapy experiments demonstrated that ligand functionalized liposomes improved gene expression[81].

2.4.3 Intracochlear delivery

Intracochlear delivery represents a promising alternative to intratympanic delivery for local inner ear therapy, by bypassing the round window membrane. The first methods for direct intracochlear drug delivery in the guinea pig were developed by Bobbin and colleagues in the early 1970s, using syringe pump infusion systems coupled to a catheter placed directly into the perilymph through a small surgical cochleostomy[82]. This technique was later extended to the mouse model [83]. When conducted carefully, the intracochlear infusion technique has little effect on neural potentials, measured by electrophysiology [84]. The first investigation of intracochlear drug
delivery has recently been conducted with gene therapy in humans and further investigation is underway [85].

The advent of chronic intracochlear drug delivery began with syringe-pump-based [86] and osmotic pump [43] technologies, the latter of which could be mounted on the animal’s head to allow ambulation during or between infusions. The osmotic pump-based system has been used to deliver antioxidants [87], monoclonal antibodies [88], neurotrophic factors [89], insulin-like growth factors [90], viral vectors [91], and drug-loaded nanoparticles [92] to the inner ear. Osmotic pumps operate by the establishment of an osmotic gradient that drives drug solution out of a canister and, in the case of intracochlear systems, through a catheter and into the perilymph. The chief advantages of osmotic pump systems are miniaturization, cost efficiency, ease of operation, and accommodation of various drug payloads [93].

Advances in the field of MicroElectroMechanical Systems (MEMS) are paving the way toward more sophisticated intracochlear infusion devices, which in contrast to osmotic pumps, can be used to control flow profiles into the cochlea in real time. The small volume of the inner ear fluids, combined with the tortuous architecture of the cochlea presents a formidable challenge to delivery systems, especially considering the requirement for adequate mixing and distribution. The aforementioned intracochlear infusion systems are limited by the low rate of clearance of cochlear fluid, and therefore can only deliver relatively small volumes in a given timeframe. Our group has previously utilized a reciprocating microfluidic pump assembly to provide higher flow rates with a zero-net volume disturbance[94-96]. Further, the use of a single cannula for both infusion and withdrawal may reduce biofouling by maintaining positive pressure at the outlet during both parts of the reciprocating cycle. Safety to hearing has been demonstrated in animal models, where DPOAEs were not significantly affected by the surgery or device activation, and CAP threshold
Elevations induced by DNQX were reversible [94]. Borkholder and colleagues have developed a modification of the murine infusion model by Chen et al, utilizing a canalostomy drilled in the posterior semicircular canal that allows for effluent flow [97]. This directed perfusion method significantly improves longitudinal cochlea flow from the base to apex, compared with that obtained with cochleostomy alone; this is presumably achieved by reducing fluidic resistance along the delivery path. Future efforts in developing the system include further miniaturization of microfluidic components, and detailed mathematical modeling of convective and diffusive drug transport to optimize delivery parameters.

In the mid 1980’s, the FDA approved cochlear implants for use in adults, and shortly afterwards approved implantation in young children. Cochlear implants work by means of a linear electrode array that is surgically inserted into the cochlea via a cochleostomy [98], or directly inserted through the RWM [99]. As the electrode array is advanced, it coils with the cochlear cavity along the peripheral tonotopic axis; insertion depth from the cochlear base varies widely, ranging from 16 mm to 31.5 mm in one study [100]. Since the prosthesis is implanted directly into the cochlear fluids, it provides a very convenient way to deliver drugs in these patients. The approaches for drug delivery utilizing a cochlear implant are active infusion pumps integrated with the electrode assembly, or drug-eluting polymer coatings. The impetus behind prosthesis-mediated drug delivery technologies is primarily to ameliorate damage caused by the insertion of the electrode itself. Local delivery of steroids in guinea pigs reduces auditory trauma during surgery [101]. In one instance, the curing behavior of silicone rubber in the presence of dexamethasone and dexamethasone phosphate was investigated [102], and in vitro release studies demonstrated sustained dexamethasone release from a silicone-coated device with a linear electrode geometry [103, 104]. Preclinical safety studies in guinea pigs have shown that cochlear-implant electrodes
with dexamethasone-eluting silicone coatings do not increase the risk of post-surgical otogenic meningitis when compared with control electrodes [105]. Other polymer electrode coatings have been developed for extended release, such as hydroxy-ethyl-cellulose [106]. Electrode coatings have been investigated for delivery of neurotrophic factors, including BDNF [107] and neurotrophin-3 [108], which promote SGN survival and neural outgrowth toward the electrode array[109]. While electrode coatings improve delivery to the apical cochlear regions, they lack active control over dosing parameters. A laser machined implant, with delivery ports along the electrode array, takes full advantage of implant insertion depth and enables on-demand delivery when coupled with an external delivery pump [110]. Likewise, a conducting polypyrrole coating was investigated for active control of dosing, and exhibited greater neurotrophin release when electrically stimulated [108].

Implantable drug delivery systems for treatment of inner ear diseases are being developed in response to recent advances in molecular biology and understanding of mechanisms of hearing loss, progress in miniaturization and integration of microdevices, and a need for new tools for use in preclinical models for drug development. In addition to these advances in microdevice fabrication and in therapeutics development, significant progress in understanding basic mechanisms of drug transport within inner ear structures has been realized. Comprehensive pharmacokinetic models of drug transport in the cochlea have been developed[54, 111, 112], encompassing a broad array of transport mechanisms in the inner ear[70]. These models provide a powerful tool for designing drug delivery approaches capable of achieving sustained, high concentrations of drug in the cochlea, accounting for important clearance mechanisms. The effects of drug binding have also been incorporated into pharmacokinetic models, further expanding the utility of these approaches[113]. Ultimately, microscale drug delivery systems will serve as a
means to deliver drugs efficaciously and safely to the cochleae of patients suffering from a range of disorders including hearing loss, balance deficits and tinnitus. However, the first use of these devices more likely resides in their application as a tool to probe mechanisms of drug effects and to evaluate efficacy and safety in preclinical animal models.

Direct surgical access to the cochlea is not a new idea, as it has been investigated extensively in the development of cochlear implant procedures over the past several decades. Therefore, the risks associated with existing clinical procedures that directly violate the inner ear are known. In particular, methods of electrode array placement through a drilled cochleostomy or insertion through the RWM have been evaluated [114]. Placement of the electrode array often results in significant damage to the endogenous structures of the cochlea, including rupture of the basilar membrane and Reissner’s membrane [115]. Although these complications do not necessarily interfere with cochlear implant performance, they would almost certainly outweigh any potential therapeutic benefits in an ear with significant residual function, and therefore should be avoided when establishing access for a local drug delivery system. Stapedectomy is a surgical procedure to mobilize a sclerotic stapes in an otherwise functioning inner ear and involves structures in direct contact with the inner ear fluids. Stapedectomy carries a 0.6-3% chance of complete SNHL in the operated ear, due to rupture of Reissner’s membrane, labyrinthitis, or other unknown causes[116]. An additional consideration for surgical entry to the cochlea is the relatively rare overpressure provided by the CSF in patients with a patent and enlarged cochlear aqueduct, which can lead to a fluid “gusher” upon perforation[117]. Other considerations include avoidance of acute infections caused by inner ear surgery and minimization of the inflammatory and fibrotic response that accumulates over time in many electrode implanted ears[118]. Therefore, the risk-benefit ratio will
determine if the more invasive intracochlear technique is justified as a treatment option for various inner ear disease.

While the goal of intracochlear drug delivery systems is for human clinical use in treatment of SNHL, NIHL and other auditory and balance disorders, the most likely near-term application of these technologies lies in their use for pre-clinical models. The development of new therapeutics, and establishment of their safety and efficacy, has been gated by an absence of reliable, practical means to deliver drugs to the cochlea and to obtain accurate measures of drug concentration and drug effects. While passive release technologies may offer the ability to reach the cochlea through relatively simple delivery modalities, they do not provide well-controlled delivery kinetics or the ability to make measurements of drug concentrations in real time. Microdevices with advanced flow control and sensing systems provide both capabilities and may prove to be the most effective near-term approach for pre-clinical studies in which a high degree of control over delivery parameters and a means to sample and measure drug concentrations in the perilymph during the delivery process are required [119]. As drug delivery microsystems continue to evolve through further miniaturization and integration of functionality, wearable and ultimately implantable devices will begin to become available for human clinical use, representing a breakthrough in the treatment of these heretofore intractable and devastating conditions.

2.5 Assessing pharmacokinetics in the cochlea

Animal models play a critical role in the research and development of new therapeutics and biomedical technologies, and this is certainly also true for intracochlear delivery devices. The aim of intracochlear delivery technologies is to provide improved spatiotemporal control of drug concentrations within the cochlea. Therefore, critical to the assessment of new inner ear delivery devices is the ability to measure drug distribution in the cochlea. Figure 2.6 shows the fluid
compartments of the cochlea. It is within the scala tympani compartment, where drugs are locally applied and gain access to the sensorineural cell populations, that the distribution profile should be measured.

![Cochlear fluid compartments](image)

**Figure 2.6 Cochlear fluid compartments.**

Left, image of the decalcified guinea pig cochlea, showing the four cochlear turns from base to apex. Right, schematic of the parallel fluid compartments, showing the position of the round window membrane (RWM), the cochlea aqueduct (CA) and the organ of Corti (orange).

The emergence of many promising drugs for sensorineural hearing loss has drawn attention to the importance of drug delivery and pharmacokinetic approaches in the cochlea.[120, 121] It has been shown that intratympanic delivery, the clinical standard of care for local therapy, results in substantial base-apex drug concentration gradients in the cochlea, due to the round window membrane (RWM) transport barrier and rapid clearance from the base of scala tympani.[54, 122, 123] Advanced delivery devices aim to maintain concentrations within the therapeutic window and improve drug distribution along the cochlear axis. However, quantitative pharmacokinetic assessment in the inner ear is required to evaluate duration and distribution of bioavailable drug
substances along the spiral axis of the cochlea.[124] Spatio-temporal measurement of drug levels within the organ of Corti is of particular interest since it contains the sensorineural cell targets for hearing protection and regeneration therapy. However, traditional approaches to pharmacokinetic analysis are very challenging in the cochlea, primarily due to its tiny fluid volume and anatomical isolation within the temporal bone and, to our knowledge, there are no reports of direct quantification of drug levels inside of the organ of Corti.

2.5.1 Fluid sampling

Multiple methods of measuring drug levels in the cochlea’s perilymphatic fluid have been established. By far the most widely used technique is sampling of the scala tympani (ST), which can be performed at the cochlear base or apex. Perilymph samples collected from the base are often significantly contaminated with cerebrospinal fluid (CSF) that enters the ST via the cochlear aqueduct during withdrawal of perilymph [125, 126]. An apical sampling method has been demonstrated to provide uncontaminated perilymph samples in approximately four sequential aliquots from apex to base in live guinea pigs [127, 128]. Other perilymph measurement approaches include microdialysis and ion-selective electrodes, which have been used to measure drug concentration as a function of time at a few discrete locations in the scala tympani [129, 130]. Despite the utility of these approaches for quantifying solute kinetics in the perilymph, they do not provide a direct measurement of how much drug reaches the target cell populations in the cochlea’s sensory epithelium. Perilymph samples also only reflect instantaneous drug levels, and do not provide a measure of cumulative distribution. Furthermore, spatial resolution is limited by the minimum size of the perilymph samples taken, where smaller samples are increasingly difficult to manage and unfavorably increase the lower limit of detection by standard analytical chemistry techniques.
2.5.2 Inferring distribution of neuroactive substances with electrophysiology

Functional assessment of hearing, utilizing well-established physiological parameters, provides a powerful means to assess pharmacokinetics and efficacy of intracochlear drug delivery devices. Two commonly used tests are the auditory brainstem response (ABR) and the distortion product otoacoustic emission (DPOAE). The ABR provides information about the magnitude of the neural response to an acoustic stimulus, and is used clinically to diagnose retrocochlear disease, whereas the DPOAE probes hair cell function and is used clinically for numerous purposes. In the context of preclinical evaluation of drug delivery systems, monitoring of the ABR as a function of sound frequency is particularly useful for assessing pharmacokinetics. When certain ionotropic receptor antagonists, such as 6,7-dinitroquinoxaline-2,3-dione (DNQX), are administered, ABR measurements at decreasing frequencies can give an estimate of how far the drug has travelled along the tonotopic axis (Figure 2.7) [94]. On the other hand, DPOAEs are not sensitive to DNQX, and are useful as a control to provide an estimate of damage produced by during surgery, by measuring OHC motility [131].
2.5.3 Fluorescence

Recently, the distribution of fluorescently labelled bisphosphonates after systemic, intratympanic, or intracochlear delivery has been quantified by fluorescent imaging of the mid-modiolar axis, which has been exposed by drilling away half of the calcified cochlea.[132, 133] Bisphosphonates bind strongly to bone, and the level of fluorescence in the lateral wall and modiolus is quantified at each turn (Figure 2.8). Unfortunately, this method used for bisphosphonate trafficking is not generalizable, as most compounds do not accumulate on the bone or the surrounding tissue.

Figure 2.7 DNQX suppresses the CAP Threshold with no effect on DPOAE [94]
However, the use of fluorescence to quantify drug distribution along the cochlear axis, motivated us to investigate the use of fluorescent substances that accumulate in the sensorineural cells.

![Figure 2.8 Fluorescent bisphosphonate labelling of the cochlear capsule](image)

In summary, significant advancements in both inner ear drug delivery and pharmacokinetic assessment in the cochlea have been made over the last two decades, but much work remains. Specifically, methods of measuring drug delivery to the target cell populations with high spatial resolution and robust quantitation techniques need to be established to evaluate and compare the various proposed strategies of intracochlear delivery. The translation of novel intracochlear delivery approaches will require a demonstration of substantial improvements over less invasive approaches, such as systemic or intratympanic delivery to justify the increased level of invasiveness.
References


3  Computational pharmacokinetic modeling in the scala tympani

3.1  Introduction

The inner ear represents a tortuous labyrinth of tiny, unstirred fluid compartments. The cochlea is unique from other luminal spaces in the body, because of the extremely low rates of convection, and, thus, the dependence on diffusion for the transport of solutes within the fluid compartments [1]. This creates a challenge for drug delivery, where even distribution of delivered substances, along the cochlear axis, is typically desired. The clinical standard for local drug delivery to the cochlea is intratympanic delivery, where an aqueous drug solution is introduced into the middle ear by injection with subsequent absorption into the inner ear, primarily via the round window membrane [2, 3]. However, the limited permeability of the round window membrane [4], the variability in round window membrane thickness observed in the pathological human cochlea [5], and the limited contact time of the drug solution with the round window membrane have led to an increased interest in intracochlear delivery, where substances are injected directly into the base of the scala tympani [6].

Over the past several decades, the guinea pig has been the most widely used animal models for the study of intracochlear drug delivery [7, 8]. In most of these studies, drug solutions were injected directly into the scala tympani compartment through a cochleostomy opening made in the bone near the base of the scala tympani compartment [9]. During injection, often controlled by an external syringe pump, fluid flows from cannula tip towards the base where there is an efflux route through the cochlear aqueduct (Figure 3.1)[10]. Therefore, after a brief injection there is a high concentration of the drug in basal region of the cochlea. However, how drug distributes after the injection is complex and difficult to assay directly. A technique of perilymph sampling in the
guinea pig can be used to collect sequential samples from the apex towards the base, but is limited greatly in spatial resolution [11]. Our group has previously made use of surrogate drugs with glutamatergic activity together with electrophysiology to infer the concentration of drug along the tonotopic axis [12]. However, this approach is limited in that it does not provide a direct measure of drug concentration and only compounds with glutamatergic activity can be detected.

Computational models provide great utility for analyzing and extrapolating the limited spatiotemporal data that can be measured for intracochlear drug concentration [13, 14]. However, the modeling approach must carefully take into account the delivery protocol used, the anatomy of the animal model chosen, and careful assignment of parameters must be obtained from literature or from parameter fitting with experimental data. However, design of the model must be matched to the delivery protocol used.

Here we present a 1-dimensional finite-difference model of drug transport in the scala tympani compartment, with parameters for the guinea pig cochlea obtained from the literature. Dexamethasone pharmacokinetics have been studied extensively in the guinea pig model, and therefore we chose dexamethasone as a representative small molecule solute for our model. We implement this model to predict spatiotemporal solute concentrations along the scala tympani for both a rapid intracochlear delivery protocol, described further in the methods, near the base of the scala tympani and intratympanic delivery. The model results allow a comparison of drug distribution, achieved by intratympanic versus intracochlear delivery, and provide the basis for the comparison of drug distribution for interpretation of in vivo intracochlear tracer studies presented in later chapters. Finally, adapting the model for sustained intracochlear delivery provides the rationale for the design of sustained-release intracochlear delivery vehicles.
Figure 3.1 The guinea pig cochlea

Left, dissection microscope image of a decalcified guinea pig cochlea showing the 4 cochlear turns between the round window membrane (RWM) and the cochlear apex. Right, schematic of the three fluid compartments showing the three scalae, the round window membrane, cochlear aqueduct, and the organ of Corti (orange).

3.2 Quantitative Methods

3.2.1 1-D mathematical model of the guinea pig cochlear perilymph compartments

The perilymphatic compartments of the cochlea (scala tympani and scala vestibuli) were modeled as a continuous fluid-filled tube, with the base of the scala tympani defined as $x = 0$ (Figure 3.2). The lengths of the scala tympani and scala vestibuli in the guinea pig are 1.62 cm and 1.56 cm,
respectively [15]. Diffusion is the dominant driving force for solute transport within the cochlea fluids, and diffusive transport within the tube model is expressed as Fick’s Second Law:

\[
\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \quad \text{for} \quad 0 < x < l_{ST} + l_{SV}
\]

where \(D\) is the diffusion coefficient of the solute in perilymph, and \(c\) is the perilymph solute concentration. The implicit assumption in this tube model is that drug only enters the scala media from the scala tympani by axial transport along the diffusion path, rather than by radial transport into this adjacent fluid compartment. The diffusion coefficient for solute molecules is typically approximated by the Stokes-Einstein equation for the diffusion of spherical particles through a liquid in the low Reynolds number regime:

\[
D = \frac{k_B T}{6 \pi \eta r}
\]

where \(k_B\) is Boltzmann’s constant, \(T\) is the absolute temperature, \(\eta\) is the dynamic solution viscosity, and \(r\) is the radius of the spherical solute. The diffusion coefficient of a small molecule drug in perilymph is assumed to be identical that in water, which is on the order of \(10^{-5}\) cm\(^2\)/s.

Before including the cochlear aqueduct in the model, the no flux boundary condition was implemented at the base of the scala tympani:

\[
D \frac{\partial c}{\partial x} = 0 \quad \text{for} \quad x = 0
\]

The termination of the scala vestibuli, which opens into the larger vestibule, was modeled as an infinite solute sink:

\[
c = 0 \quad \text{for} \quad x = l_{ST} + l_{SV}
\]
3.2.2 Radial clearance from scala tympani

Drug loss by radial clearance, representing elimination mechanisms to the vasculature and adjacent fluid or tissue compartments, were modeled as a first-order reaction along the diffusion path:

\[
\text{clearance rate} = -k c_x(x, t)
\]

where \( k \) represents the first-order rate constant, modeled as being uniform along the tube, and defined as:

\[
k = \frac{\ln(2)}{\tau}
\]
Where \( \tau \) is the characteristic elimination time (half-time), which was assigned 90 minutes, based on a published quantitative study of literature reports of steroid clearance from the scala tympani [16].

### 3.2.3 Correction term to 1-D diffusion equation for changing ST cross-sectional area

A correction term for the changing cross-sectional area was added to the 1-D diffusion equation to account for the 3-D geometry of the compartments [17]:

\[
\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} + \frac{D}{A_x} \frac{\partial A_x}{\partial x} \frac{\partial c}{\partial x}
\]

where, \( A_x \) is the cross-sectional area of the scala tympani (or scala vestibuli) at distance \( x \) from the base of the scala tympani. The cross-sectional areas between \( x = 0 \) and \( x = l_{ST} + l_{SV} \) implemented in the model are shown in Figure 3.3.
3.2.4 Solute clearance via diffusion through the cochlear Aqueduct

The cochlear aqueduct was modeled as an extension of the tube at the base of the scala tympani ($-l_{CA} < x < 0$). In the guinea pig the cochlear aqueduct is about 1.5 mm long and has a cross a cross-sectional area of approximately 0.02 mm$^2$ [18]. When the cochlear aqueduct was included in the model, the no flux boundary condition, at $x = 0$, was removed and replaced by an infinite solute sink at the Arachnoid space-end of the cochlear aqueduct:

$$c = 0 \quad \text{at} \quad x = -l_{CA}. $$
3.2.5 Initial conditions for intracochlear delivery

For this model, we assumed intracochlear drug delivery was achieved by a bolus injection of a 2-µL concentrated drug solution into the base of the scala tympani via a microfluidic cannula terminating 5 mm from the base of the scala tympani. During the injection, drug solution is assumed to flow towards the base and out through the cochlear aqueduct to accommodate the volume change. The concentration basal to the cannula tip is assumed to be uniform due to both the degree of mixing affected by the induced convection and the relatively short diameter of the scala tympani (approximately 0.6 mm near the cannula tip) compared to the substantially longer axial diffusion distance along the scala tympani (16 mm). Therefore, the injection is modeled as a step increase, representing a relatively short infusion period, in drug concentration between the tip of the injection cannula and the Arachnoid space- end of the cochlear aqueduct of the scala tympani:

\[
\frac{\partial c}{\partial t} = 0 \quad \text{for} \quad x = l_{C,A.} \quad \text{and} \quad x = l_{cannula}
\]

\[
c = c_o \quad \text{for} \quad -l_{C,A.} < x < l_{cannula}
\]

\[
c = 0 \quad \text{for} \quad l_{cannula} < x < l_{ST} + l_{SV}
\]

where \(c_o\) is the initial solute concentration in the base of the scala tympani and cochlear aqueduct after the injection.

3.2.6 Initial and additional boundary conditions for intratympanic delivery

For intratympanic delivery, there is no solute assumed to be in the cochlear fluids at the time that drug is introduced into the middle ear, since there is no direct access to the perilymph:

\[
c = 0 \quad \text{for} \quad t = 0 \quad \forall x
\]
Transport through the round window membrane is modeled as a constant flux determined represented as the product of the permeability of the round window membrane to the solute $P_{RW}$ and the applied middle ear solute concentration $C_{ME}$:

$$J_{RW} = P_{RW} \cdot A_{RW} \cdot t \cdot C_{ME}$$

where $J_{RW}$ is the total amount of solute that has crossed the round window membrane at time $t$; and $A_{RW}$ is the area of the round window membrane – approximately 1 mm$^2$ in the guinea pig. The permeability of the round window membrane, $P_{RW}$, was assigned to the reported permeability of the intact guinea pig round window membrane to dexamethasone ($3.5 \times 10^{-8} m/s$) [19].

3.2.7 Implementation of the finite difference method in MATLAB

The governing equations for the spatiotemporal drug distribution inside the scala tympani compartment of the guinea pig cochlea, described here, were solved using the 1-dimensional finite-difference method (central-difference method), $\Delta x = 0.001 cm$ and $\Delta t = 0.01 s$, implemented in MATLAB®. For all simulations conducted, the conditions for von Neumann stability, a method of checking numerical stability[20], were met, and maximum relative errors in mass conversation were below $10^{-10}$.

3.3 Results & Discussion

3.3.1 Intracochlear delivery

The effects of radial clearance, cross-sectional area, and diffusive clearance through the cochlear aqueduct on the spatiotemporal distribution following a bolus intracochlear injection were each considered. Radial clearance, alone, results in substantial reduction of solute concentration within
the first hour after delivery, with the greatest effect basal to the injection site, where there is the highest initial drug concentration Figure 3.4, left. The radial clearance has less impact of the penetration depth of drug concentration within the first hour, compared to a simple diffusion model without any clearance. The narrowing of the cross-sectional area from maximum area towards the apex slightly increases the concentrations due to the concentrating effect of drug diffusion into smaller fluid volumes Figure 3.4, middle. Adding the cochlear aqueduct as a diffusion efflux route to the Arachnoid-space diffusion sink modestly reduces the concentrations throughout the length of the cochlea by reducing the basal concentrations and the base-apex gradient, which is the driving force for apical diffusion Figure 3.4, right. These results at 1-hour post injection are qualitatively similar to data from a literature report of a model fit to sequential samples collected from the cochlear apex 40 minutes after an injection of dexamethasone solution into the cochlear base [9].

Figure 3.4 Effect of model components at 1 hour after I.C. bolus injection

Concentrations relative to the initial concentrations, basal to the cannula tip, are shown as a function of distance from the base of the scala tympani compartment. Dashed lines show the model results without the indicated model component (solid lines). Arrows indicated the site of the tip of the cannula insertion depth.
The rapid rate of drug clearance and diffusion results in decrements basal to the injection site, over an order of magnitude, within the first 6 hours after delivery (Figure 3.5). The drug concentration in regions apical to 10 mm from the base increases up to 3 hours after delivery, while there remains a base-apex concentration gradient, but falls again within 6 hours after delivery, when clearance overcomes the base-apex concentration gradient. This suggests that for effective therapy of a drug solution, with solute clearance rates comparable to dexamethasone, the drug must either have wide therapeutic window and must be injected multiple times within several hours in order to maintain drug levels for the duration of therapy.

Figure 3.5 Spatiotemporal drug distribution after I.C. bolus injection

Relative concentration profiles at various times after injection (dashed lines) are shown against the initial condition following the bolus injection (gray).
3.3.2 Effect of cochlear aqueduct size on ICDD

In humans the diameter of the cochlear aqueduct is variable, and can differ between individuals by over a factor of 10 [21]. To evaluate the effect of possible variability in the guinea pig model we varied the cross-sectional area of the cochlear aqueduct between 0.02 mm² and 0.15 mm², and reported the distributions of relative concentrations at 1 and 3 hours after the bolus injection, Figure 3.6. Enlargement of the cochlear aqueduct led to modest reductions in concentrations in the basal regions of the scala tympani but had less effect towards the apex and did not alter the drug penetration depth at either time point. However, this model only accounts for simple diffusion through the cochlear aqueduct, and other homeostatic mechanisms are likely to be present, such as a slow apically driven flow from the cochlear aqueduct towards the apex, and a high frequency reciprocating convection at the interface between the cochlear aqueduct and the scala tympani [9].

![Figure 3.6](image)

**Figure 3.6 Effect of cochlear aqueduct area after I.C. bolus injection**

Relative concentrations plotted for several different cochlear aqueduct areas at 1-hour (left) and 3 hours (right) after I.C. bolus injection.
3.3.3 Intratympanic delivery

To compare the spatiotemporal distributions between intracochlear bolus and intratympanic delivery, we used the same mathematical framework to analyze the intracochlear drug concentrations after continuous delivery at the round window membrane. Continuous flux through the round window membrane resulted in a monotonic increase in solute concentration at all distances from the base of the scala tympani between 1 and 5 hours, Figure 3.7. However, the concentration profile reached a steady state after 6 hours, when the diffusive gradient was balanced by elimination. The maximum drug concentration was three orders of magnitude less than the concentration in the middle ear and occurred at the very base of the scala tympani, where the round window membrane is located, and the cross-sectional area is smallest. The equilibrium concentration gradient between 0 and 10 mm from the base of the scala tympani is approximately an order-of-magnitude difference. This is in qualitative agreement with analyses of solute entry through the round window membrane, which also show that only a small fraction of the middle ear concentration is attained inside the cochlea and with a substantial base-apex gradient [4].
Figure 3.7 Model spatiotemporal distribution for intratympanic delivery
Relative concentrations plotted vs. distance from base of the scala tympani at 1-hour increments (black lines). Gray band indicates and width of the round window membrane, where solute flux occurs.

The effect of the cochlear aqueduct area on intratympanic delivery was also evaluated. Enlargements of the cochlear aqueduct had the greatest effect very close to the base of the scala tympani within 1 hour, but had a larger impact on the equilibrium distribution, where reductions in drug concentration were observed within approximately the first 6 mm from the base of the scala tympani, Figure 3.8.
3.3.4 Sustained intracochlear delivery

The possible improvement in drug distribution by sustained intracochlear delivery was evaluated by enforcing constant concentration below the injection site. This would represent, for example, repeated intracochlear injections at frequencies that overcome elimination rates. When the drug concentrations are maintained in the basal region of the cochlea, below the tip of the cannula insertion, the concentrations increase at all apical locations, over the first several hours Figure 3.9. Equilibrium is reached for all distances from the base of the scala tympani after about 5 hours. The equilibrium concentration gradient from base to apex is over 1 order of magnitude, however, relative drug levels in excess of .04 can be achieved even at the apical-most end of the scala tympani.
3.4 Conclusions

We have shown, using a computational model for dexamethasone, that a single intracochlear injection results in substantially higher intracochlear concentrations compared with the maximum concentrations achieved by intratympanic delivery. For a single intracochlear injection, rapid clearance results in substantial loss of the drug within the first several hours, precluding prolonged therapy. For intratympanic delivery, only a small fraction of the concentration applied to the middle ear is available inside the cochlea, and it takes approximately 6 hours before the maximum levels are reached. Therefore the clinical standard of 1 hour of RWM contact time should be re-evaluated. For sustained intratympanic delivery, for example with an injectable polymer gel, maximum concentrations can be reached, but still will suffer an order of magnitude base to apex concentration gradient. The concentration gradient can be reduced if a prolonged intracochlear
delivery approach is utilized, for example by repeated intracochlear injections. With sustained intracochlear delivery, reasonable drug concentration (4% of the injected concentration) will reach even the most apical regions of the cochlea within approximately 5 hours. It is important to note, however, that the equilibrium concentrations depend heavily on the time constant for radial clearance. Here we present simulations with the time constant for dexamethasone, based on literature, but the clearance rate for other solutes would be expected to vary based on physicochemical properties, including size and lipophilicity.
References


4 Intracochlear drug delivery: Fluorescent tracer evaluation for quantification of distribution in the cochlear partition

This chapter is currently in submission by A.M. Ayoob et al.

The measurement of drug distribution in the inner ear has important ramifications for design of methods of local drug delivery, such as intracochlear delivery, and for the assessment of emerging drug candidates in preclinical animal models. In this work, we investigate several fluorescent surrogate drug tracers- FITC-dextran, Qtracker™ 655, gentamicin Texas-Red, and FM 1-43 FX, and measure their distribution following injection into the scala tympani by quantitative fluorescence imaging of dissected cochlear sections. Different patterns of fluorescent tissue labeling were observed in the basal region of the cochlea for each of the tracers, with gentamicin Texas-Red (GTTR) and FM 1-43 FX serving as promising sensory cell specific labels. The fluorescence signals for these two tracers were localized to the hair cells of the organ of Corti. Spatial distributions of tracers were inferred from measurements of fluorescence intensity, with relatively high spatial resolution afforded by confocal microscopy. Results of this investigation demonstrate that certain fluorescent tracers may serve as drug surrogates toward the development of new drug delivery strategies for the inner ear, particularly for intracochlear approaches that aim to improve drug distribution.

4.1 Introduction

Research advancements in the pathophysiology of hearing loss and the identification of molecular targets for restoring cochlear function are accelerating the discovery of a range of potential pharmacological treatments. Treatment strategies include delivery of agents that protect against
ototoxicity or noise-induced damage, and regenerative strategies that aim to restore lost sensorineural cells, which function to transduce acoustic stimuli into afferent neural signals and are located in the cochlea’s organ of Corti [1, 2]. Included in this group of emerging drugs are apoptosis inhibitors and antioxidants that prevent sensory hair cell death after injury [3, 4], neurotrophic factors that promote neurite survival and outgrowth [5-8], gamma secretase inhibitors that induce differentiation of supporting cells into new hair cells [9], and an array of gene therapies targeted at forms of congenital deafness [10].

The effectiveness of emerging therapies requires drugs to be delivered within a therapeutic window, which must be first measured in preclinical animal models before advancement to human use [11, 12]. For most substances, systemic delivery is impeded by the blood-cochlea barrier and poses the risk of off-target side effects [13-15]. Intratympanic (IT) delivery, while circumventing the systemic circulation by injection into the middle ear, is often associated with limited absorption [16, 17], and poor drug distribution to the mid and apical regions of the cochlea [18]. Intracochlear drug delivery (ICDD) entails direct administration of drugs into the cochlear fluids, enabling improved precision over dosing pharmacokinetics and the potential for well-controlled spatiotemporal drug distribution [19, 20]. Drug solutions can be administered directly to the perilymph of the scala tympani by insertion of a cannula through the round window membrane (RWM) or a cochleostomy opening made near the base of the cochlea (Figure 4.1).

A principal advantage of ICDD compared to systemic or IT delivery is direct access to the cochlear fluids and the spiral sensory epithelium, a benefit which must be balanced against the invasive nature of surgical access to the cochlea. An early ICDD approach utilized a cannula surgically inserted through a cochleostomy near the RWM, with flow rates metered by an external syringe pump[21]. Alternative approaches utilized wearable osmotic pumping systems that were aimed at
stable delivery rates for several weeks[22], and has been widely used in preclinical animal models for the study of pharmacologic effects in the cochlea[23-26]. More recently, advances in microelectromechanical system (MEMS) technologies have been leveraged toward a wearable reciprocating delivery device that exhibits zero-net volume delivery with a chronic system comprising a micropump delivering compounds from an onboard drug reservoir[27, 28]. In all of these systems, delivery near the base of the cochlea result in a concentration gradients from base to apex [29], which can be addressed by extended delivery sequences, improved mixing, or by the surgical introduction of an efflux route to direct convection from the injection site at the base towards cochlear apex [30].

Translation of ICDD approaches to human use will rely on accurate measurement of drug distribution and pharmacokinetic assessment in preclinical animal models. Several methods have been established to assay drug concentration in the cochlea following delivery. The most widely used approach is perilymph sampling, which can provide very accurate levels of drug concentration within the scala tympani but has limited spatial resolution, especially towards the apical regions due to the ST narrowing [18]. Other methods, such as micro CT imaging, are capable of measuring spatial distribution of solute but are limited to radio-opaque tracer substances that may not reflect the pharmacokinetics of various drug molecules of interest [31]. Another common assay is provided by electrophysiological measurements to characterize intracochlear distribution of substances with glutamatergic activity, such as 6,7-dinitroquinoxaline-2,3-dione (DNQX), which provides tonotopic mapping of drug distribution as a function of hearing frequencies. Compound action potentials (CAP) and distortion product otoacoustic emissions (DPOAE) can be employed to assess specific information regarding drug effects versus other mechanisms such as damage via threshold shift or amplitude levels at various frequency locations [32]. However, one
of the limitations with this technique is limited dynamic range for inferring DNQX concentration from the electrophysiological measurements, owing to the nature of the dose-response curve for DNQX. Another promising approach for measuring drug distribution is the use of fluorescently-labelled drug conjugates, whose concentration can be measured with fluorescence microscopy after delivery and cochlear dissection. For example, the delivery of fluorescently-labelled bisphosphonates, which bind bone with high affinity, was assessed at each half-turn by fluorescence imaging of the mid-modiolar section [33, 34].

Cochlear sensory hair cells and the spiral ganglion neurons fibers are located within the organ of Corti, which spirals along the cochlear axis. The organ of Corti can be dissected and prepared using whole mount techniques and imaged from base to apex. This approach has been widely used to quantify the distribution of sensorineural damage by counting hair cells[35], nerve fibers [36], and synaptic terminals [37] along the organ of Corti. However, the potential utility of whole mount dissection and imaging for quantification of drug distribution has not been investigated; yet, this tissue might represent the most relevant place to measure drug distribution, as it contains the cells that are the targets for most emerging drugs. We hypothesized that quantification of fluorescence signal in fixed tissue sections of the organ of Corti could provide a measure of spatial distribution for certain fluorescent drug-surrogate tracer substances, delivered \textit{in vivo} to the scala tympani compartment. For substances administered to the base of the cochlea, a large baso-apical gradient is expected, due to the diffusion-limited transport towards the apex [38]. We screened a small array of fluorescent substances as candidate tracers, by evaluating their ability to label tissue near or within the organ of Corti and measuring their distribution along the cochlear axis after delivery to the base of the scala tympani.
Tracer candidates were selected on the basis of their predicted affinity for various cell populations after intracochlear delivery to the scala tympani. The array of tracers selected consisted of FITC-dextran, with little expected affinity to any cell type in the cochlea [39]; Qtracker™-655, which is efficiently internalized by many different cell types in culture [40]; gentamicin-Texas Red, which is specific for hair cells when injected systemically [41], and has shown some specificity for the apical cytoplasm of the OHCs when delivered locally to the RWM [42]; and FM 1-43 FX, a fixable analogue of FM 1-43, which is efficiently internalized by inner hair cells at both their apical and basolateral surfaces [43, 44]. In measuring the distribution of these different candidates, we systematically applied several quantification methods, to discriminate differences in labelling patterns between candidates.

4.2 Methods

4.2.1 Animals

Male Hartley albino guinea pigs (Charles River Laboratories, Inc., Wilmington, MA) weighing 300-500 g were used in this study. All procedures were approved by the IACUC of the Massachusetts Eye and Ear Infirmary. Ketamine (30 mg/kg, IP) and xylazine (2 mg/kg, I.P.) were administered as sedatives prior to anesthesia. Animals were anesthetized by isoflurane gas (1.5%, inhaled). The retroauricular area was shaved and prepared with betadine antiseptic solution. Ophthalmic ointment was applied to the eyes as needed during anesthesia. Atropine (0.04 mg/kg, S.C.) was administered to control bronchial secretion during surgery. During anesthesia, heart rate, rectal temperature, and blood oxygen levels were monitored every fifteen minutes.
4.2.2 Preparation of fluorescence surrogate drug solutions

The following solutions were prepared by dilution of each fluorescent substance in artificial perilymph [(mM) NaCl, 120; KCl, 3.5; CaCl2, 1.5; glucose, 5.5; HEPES, 20; NaOH added to bring pH to 7.5] and loaded into a 500 µL Hamilton Syringe: FITC-dextran-lysine (10 KDa, ThermoFisher Scientific) was chosen as a fixable fluorescent water soluble macromolecule and was prepared at 6 mg/mL; Qtracker™ 655 (ThermoFisher Scientific) was chosen as a quantum-dot ligand intracellular labelling system and was prepared at 100 nM; gentamicin Texas-Red (GTTR), (Dr. Peter Steyger, Oregon Hearing Research Center) was chosen as a fluorescent gentamicin conjugate known to accumulate in hair cells, and was prepared at 1 mM; and FM 1-43 FX (ThermoFisher Scientific) was chosen as a membrane probe, known to be internalized by inner hair cells, and prepared at 35 µM.

4.2.3 Surgery and intracochlear drug delivery

The bulla was exposed by dissection and opened with a blade to visualize the cochlear basal turn and the RWM. A fine drill bit was used to generate a cochleostomy (approximately 200 µm in diameter) 0.5 mm distal to the RWM. Fluorescent tracer solutions were loaded into a Hamilton Gastight 1700 Series Syringe with Luer lock, connected to Poly(etherether ketone) (PEEK; Idex Corporation) tubing, terminating in a short (4.5 cm) length of Teflon cannula (PTFE tubing with a 101µm ID, 201 µm OD). A soft silicone bleb was deposited 3 mm distal to the cannula tip, which served to both control the insertion depth and provide a seal of the cochleostomy during injection. Once the cannula was inserted into the cochleostomy, infusion was metered by a Harvard Apparatus PHD Syringe Pump (Holliston MA) at 1 µL/min for 2 minutes. After the infusion cycle, the cannula was removed and the cochleostomy was sealed with a small piece of fascia and carboxylate cement (Durelon™). The animals remained under anesthesia for a total of 3 hours
after the intracochlear infusion and were euthanized by pentobarbital overdose (Fatal-Plus) and fixation was performed by intracardial perfusion of a 4% formalin solution.

**Figure 4.1.** Intracochlear drug delivery to the base of the guinea pig scala tympani.

a) Teflon cannula is inserted through a cochleostomy opening at the base of the scala tympani compartment, near the round window membrane, and threaded 3 mm apically. The distance from the “base” of the organ of Corti (orange) is defined as $x=0$, and the closest distance to the cannula tip is approximately 5 mm. b) Surgical microscope view of the basal-most portion of the cochlear bone and the round window membrane, exposed by bullectomy (**left**). Yellow fluid (FITC-dextran) can be visualized through the translucent round window membrane and the cochleostomy opening is pictured before sealing (**right**).
4.2.4 Tissue Preparation

Cochleae were dissected from the temporal bones, post-fixed overnight in the same fixative at room temperature and decalcified in 0.12 M EDTA for 1-2 weeks. After washing with 0.1M PBS, the organ of Corti of each cochlea was micro dissected into 8-12 pieces. For each section, the tectorial membrane is removed with forceps, and the spiral ligament is trimmed with a fine blade. Sections are mounted on glass slides with Vectashield antifade mounting medium (Vector Labs). The process of fixation and washing is expected to remove any tracer that is not covalently bound by fixation or sequestered in a tissue compartment by non-covalent interactions.

4.2.5 Confocal microscopy

A Zeiss LSM 710 laser scanning confocal microscope with a 20X objective was used to image the organ of Corti in adjacent optical sections, each 2.6 µm in thickness. Sodium fluorescein solutions were used to generate calibration curves at several photomultiplier tube (PMT) gain settings. The linear range for each master gain setting was fit to a linear curve by the least-squares error method. Confocal images, acquired at 16-bit pixel depth, were analyzed using ImageJ software (NIH).

4.2.6 Quantification Methods

We systematically applied several quantification techniques that measured fluorescence distribution along the cochlear axis in different parts of the whole mount preparations, including the rows of inner and outer hair cells, the bodies of the spiral ganglion neurons, and the entire tissue section. For these analyses, sum intensity projections were generated spanning the tissue sections from the confocal z-stack. Line scans were drawn over the rows of inner hair cells and
outer hair cells, with line-widths of 25 µm and 37.5 µm, respectively (Figure 4.2, middle). For the region containing the bodies of the spiral ganglion neurons, a 62.5 µm trace was drawn. The pixels from the sum intensity image contained in these traces were averaged in bins of distance from the base of the organ of Corti using MATLAB. For quantifying the fluorescence in the entire width of the tissue sections, arcs of concentric circles were drawn around the inner and outer borders of each section in ImageJ. Area segments, 30 µm-wide at the location of the row of inner hair cells, and bound by the arcs, were drawn over the sum intensity image of the tissue section (Figure 4.2, right). The average intensity contained in each area segment was measured, and the results were concatenated along the length of the cochlea in MATLAB.

**Figure 4.2 Dissection and quantification regions on whole mount sections**

Fixed, decalcified cochleae (left) are dissected to remove the organ of Corti in sections (dotted lines show the base most section of the cochlear partition). Regions of interest are drawn over the confocal images using ImageJ to isolate the rows of
the inner and outer hair cells (middle). Arcs of concentric defining the inner and outer tissue regions are drawn, and segments are drawn between to quantify the fluorescent signal in increments along the entire tissue section (right). Sections shown here are inverted sum intensity projections of autofluorescence from a control (no injection) cochlea taken on the FITC channel.

4.2.7 Automated GTTR fluorescence in OHCs

The fluorescence signal from the outer hair cells was quantified using an automated routine designed in ImageJ (NIH) macro editor. Briefly, the optical sections spanning the hair cells (user input) were combined by max intensity projection and converted to 8-bit inverted images. A threshold (pixel intensity>200) was applied to the composite 8-bit image. ImageJ’s Particle Analyzer function was applied to 37.5-µm thick trace(identified by user input) spanning the row of outer hair cells (OHCs) and stored the location of each identified “particle” in a image mask. The image mask was applied to the original 16-bit max-intensity composite image and the max pixel intensity and the xy location for was recorded for each particle. Using Matlab, the peak intensities for each particle were averaged together in 0.5 mm increments. The density of OHCs per mm, defined by the density of “particles” above the threshold value, was also determined in 0.2-mm increments. In regions where the particle density fell to 0 (above approximately 7 mm from the base), indicating that the GTTR signal in the OHCs could not be distinguished from the background, the fluorescence intensity was plotted as the mean total signal within the user-defined trace.
4.3 Results

4.3.1 Intracochlear injection

A surgical microscope enabled visualization of the RWM and bone of the first cochlear turn through the surgical opening in the bulla. Solutions were perfused into the scala tympani perilymph through a cannula inserted into the surgical cochleostomy and advanced to an approximate distance of 4-5 mm relative to the organ of Corti (Figure 4.1, a), as described in the methods. Two microliters of drug solutions were administered over a 2-minute infusion period. When the FITC-dextran solution was injected we observed, through the translucent RWM, the yellow solution at the base of the cochlea, (Figure 4.1, b). No yellow FITC-dextran solution was observed outside of the cochleostomy either during the injection or after withdrawing the cannula, indicating that any fluid leaking from the cochleostomy was sufficiently dilute that it could not be observed visually. After each injection, the cochleostomy was sealed with dental cement, and the seal was visualized for leakage. We did not observe leakage from the cochleostomy after any of the injections.

4.3.2 Tissue preparation and imaging

After decalcification, the spiral cochlear partition containing the sensorineural epithelium is visible (Figure 4.2, left). The cochlea is bisected through the modiolus and organ of Corti is dissected in 8-12 pieces, serially, from base to apex. Serial imaging of the sections was performed on a confocal microscope with a 20X objective (Zeiss LSM 710). Adjustment of the photomultiplier tube gain enabled measurement of fluorescence with a linear dynamic range of over 5 orders of magnitude of fluorescein concentration.
4.3.3 FITC-dextran

Specimens that were fixed hours after intracochlear delivery of FITC-dextran showed scattered fluorescence (Figure 4.3) throughout the cochlear sections in the basal half of the whole mount tissue preparation. Scattered fluorescence was observed throughout the tissue boundary, with finger-like fluorescence observed in the spiral ganglion region and could represent labelling of the spiral ganglion vasculature. Total fluorescence in the tissue sections peaked within the first millimeter from the base of the organ of Corti, with a rapid drop-off apical to 1 mm. Above approximately 10 mm from the base, no signal was measured above the threshold level. There was a notable lack of intensity in the rows of inner and outer hair cells (Figure 4.3; Figure 4.7, upper-left). We did not quantify the distribution of fluorescence within the spiral ganglia, since there was no apparent labelling of the spiral ganglion neurons, themselves.
Figure 4.3 FITC dextran labelling in whole mount sections

Sum intensity projection of the basal portion of the whole mount dissection (left), and thresholding is applied to isolate the FITC-dextran labelling (middle) from the background autofluorescence, observed on the FITC channel. Distinct finger-like fluorescence is seen in the region of tissue containing the spiral ganglion neuron bodies, but not in the rows of hair cells (right).

4.3.4 Qtracker™ 655

Specimens fixed three hours after delivery of a quantum dot with a targeting peptide for non-specific cytoplasmic labelling (Qtracker™ 655) showed fluorescence in the basal half of the cochlear tissue. The pattern of fluorescence was of concentrated punctate staining throughout the tissue boundary. When the fluorescence image was overlaid with the bright field image collected using the transmitted light detector (T-PMT) setting on the confocal microscope, signal is colocalized in the xy-plane to the row of IHCs and the outer sulcus region. In individual optical slices on a plane within the row of IHCs in the z-direction, fluorescence was clearly observed in the row of IHCs (Figure 4.4). In some regions we observed clustering of fluorescence rather than diffuse puncta, which may be the result of particle flocculation. We observed for example, that flocculation occurs in artificial perilymph after several hours in vitro at the injected concentration. Fluorescence was quantified along the length of the cochlear axis (Figure 4.7, upper-right). Fluorescence throughout the entire tissue sections peaked within the first 2 mm from base of the organ of Corti and rapidly dropped more apically. The distribution of intensity in the IHCs, OHCs, and SGNs was similar to that in the whole tissue section.
Figure 4.4 Qtracker™-655 fluorescence throughout the entire tissue section

T-PMT image overlay is used to visualize the rows of hair cells (left). Diffuse staining throughout the tissue is observed, including the row of inner hair cells and the sulcus region (middle). A confocal optical section in the plane of the hair cells, captured on both the T-PMT channel and the fluorescence channel shows, fluorescence localization to the IHCs (right).

4.3.5 Gentamicin-Texas Red

Three hours after GTTR delivery, fluorescence signal in the inner hair cells, apical regions of the outer hair cells, and region of the spiral ganglion neurons (Figure 4.5, left). In the maximum intensity projection (Figure 4.5, right) there was a clear punctate labelling of the outer hair cells.
which was localized to the apical surfaces of these cells. The distribution of fluorescence throughout the entire tissue sections was elevated in the basal portion of the cochlea, below 11 mm from the base, and was mostly below the threshold value beyond this location (Figure 4.7, bottom-left). The distribution and intensity levels of fluorescence specifically in the rows of inner and outer hair cells was similar to the nonspecific labelling throughout the entire tissue. The levels of fluorescence in the region of the spiral ganglion cell bodies was higher than that in the entire tissue within the first 4 mm from the base of the organ of Corti.

The intensity distribution of peak pixel intensity per OHC showed a clear base-apex gradient, with highest fluorescence levels 3-4 mm from the base of the organ of Corti (Figure 4.8). The count of hair cells with signal above threshold was analyzed in 0.2-mm increments from base of the organ of Corti.
**Figure 4.5 Gentamicin-Texas Red fluorescence**

Sum intensity projection shows fluorescence signal in the region of the inner and outer hair cells and the region containing the fibers of the spiral ganglion neurons (left). Max intensity projection of optical sections spanning the depth of the outer hair cells for a section of the sensorineural epithelium near the base of the cochlea. Maximum intensity projection (inverted) of a section near the base of the cochlea (right, top). Pixels with signal levels above the threshold are colored red. User-defined trace was drawn over the row of outer hair cells (yellow). GTTR fluorescence is seen in the row of outer hair cells (bottom, left). The native image is converted to an inverted 8-bit image (bottom, middle) and thresholding is applied to isolate the signal from the outer hair cells within the user defined region of interest trace (bottom, right).

### 4.3.6 FM 1-43 FX

Three hours after delivery of FM 1-43 FX, cell-specific labelling was observed in the rows of IHCs and OHCs (Figure 4.6). Diffuse labelling of the cytosol of the IHCs was observed, around dark, unlabeled nuclei. Peak fluorescence was observed between 3 and 5 mm from the base of the organ of Corti, with reduced intensity in both directions from the peak (Figure 4.7, bottom-left). The distribution of fluorescence intensity in the IHCs, OHCs, and SGNs were comparable to the entire tissue section. The signal from the SGN region was consistently higher than that in the IHCs and OHCs.
Figure 4.6 FM 1-43 FX fluorescence

Sum intensity projection shows fluorescence signal in the region of the inner and outer hair cells and the region containing the bodies of the spiral ganglion neurons (Left). Max intensity projection enables visualization of peak intensity (fluorophore concentration) in the xy-plane of the z-stack (right). A clear baso-apical gradient is observed in the rows of inner and outer hair cells and the region containing the spiral ganglion neuron bodies and fibers.

4.4 Discussion

Translation of ICDD approaches to human use will rely on accurate methods for measurement of drug distribution and, ultimately, precise tools for pharmacokinetic assessment in preclinical
animal models. Fluorescence represents a robust quantitative readout that can be measured in the tissues or bone of the cochlea after local delivery. In this study, we screened several fluorescent drug surrogates whose concentration distribution in the organ of Corti after direct ICDD could be inferred through fluorescence microscopy. Here we demonstrated that these tracers can be used to quantitatively measure the distribution of drug and drug surrogates along the cochlear partition after *in vivo* delivery to the scala tympani. Fluorescence was measured with confocal microscopy in whole mount sections of the organ of Corti fixed three hours after ICDD. The demonstrated advantage of this is high resolution measurement of fluorescence intensity in tissues that spiral along the cochlear axis inside the cochlear partition, including the sensorineural cells inside the organ of Corti. Fluorescence can also be measured with very high sensitivity and wide dynamic range.
Figure 4.7 Fluorescence intensity along the whole mount preparations as a measure of intracochlear tracer distribution

Fluorescence intensity measurements for sum-intensity images for each tracer. Fluorescence is quantified in the rows of outer hair cells (OHC) and inner hair cells (IHC), the region containing the bodies of the spiral ganglion neurons (SGN), and also throughout the entire tissue section (Section). OHC, IHC, and SGN intensities are averages in 2 mm bins, measured from the base of the organ of Corti.
Fluorescent dextrans are commonly used as tracers to investigate membrane permeability, vascular structure integrity, and endocytosis[39, 45, 46]. We identified FITC-dextran as a potential label for the sensorineural cells due to its apparent internalization into outer hair cells reported by Hu et al [39]. In that study, FITC-dextran (10kDa and 40kDa) solutions were perfused throughout the scala tympani, and the percentage of outer stained outer hair cells increased from 0% near the base to 100% near the apex. Our observation, that the hair cells were not labelled by FITC-dextran could be explained our drug delivery procedure, in which high drug concentrations were limited to the basal region of the cochlea where Hu et al observed minimal internalization. However, it also raises the obvious question of why the reported FITC-dextran internalization gradient exists in the first place. In the absence of a physiological gradient corresponding to the rate of uptake, it could be that the reported increased uptake rates at the apex could simply be an artifact of the perfusion technique which produces higher shear rates at the apex, where the scala tympani narrows. Therefore, we suggest that FITC-dextran quantification in the cochlear hair cells does not represent a useful method of evaluating surrogate drug distribution following intracochlear drug delivery. However, because fluorescence was observed in other parts of the tissue sections, which was limited to the basal region, quantification of the entire tissue (Figure 4.7), appears to be a more viable approach to measuring its distribution along the cochlear axis.

Qtracker™ 655 is a cell-penetrating quantum dot system for localization in live cells and lineage tracing. The family of Qtracker™ labelling kits were appealing candidate tracers, due to their broad targeting of many cell types and their persistent and stable fluorescence labelling; however, Qtracker™ delivery to the cochlea had not been previously investigated. Here we observed broad labelling of the tissue preparations in the vicinity of the injection site near the basal regions of the cochlea. In the optical plane of the hair cells, Qtracker™ signal was localized to the row of inner
hair cells, with substantially less fluorescence in the row of outer hair cells. We also observed staining in the regions of the outer sulcus, near the spiral ligament, and the regions corresponding to the spiral ganglion neurons. However, one of the peculiarities that we noticed was particle flocculation in artificial perilymph at room temperature at the concentrations we injected. Therefore, future studies considering Qtracker™ must employ caution for where flocculation may occur.

GTTR is a gentamicin conjugate with Texas Red that has been used to study gentamicin localization to the sensory hair cells for systemic delivery. Prior studies suggest that GTTR first enters the endolymph from the systemic circulation via the stria vascularis and then enters the apical surfaces of the cochlear hair cells through the stereocilia transduction channel [47]. However, when applied locally to the round window membrane, Zhang et al reported punctate fluorescence in the apical surfaces of the outer hair cells, with more cells labelled near the RWM in the base of the cochlea[42]. This led us to believe that GTTR could be measured in the outer hair cells when applied to the scala tympani compartment by intracochlear delivery. Our observation of punctate labelling in the outer hair cells, in the vicinity of the injection site at the basal end of the cochlea, was consistent with the observations of Zhang et al. However, a method for quantifying the distribution of this OHC labelling was not previously reported.
Figure 4.8 GTTR fluorescence intensity distribution along the row of outer hair cells

Fluorescence intensities from outer hair cells are binned by distance along the organ of Corti (top). Particle count density in the user-defined region of interest trace is region of interest are reported in 0.2 mm increments (bottom). For locations were OHC signal cannot be isolated to the outer hair cells (above approximately 7 mm from the base), the average intensity within the region of interest is reported.

If GTTR reaches the endolymph, we would expect strong staining of the IHCs as well. When GTTR solution is applied to the RWM, a similar specificity to the apical surfaces of only the OHCs
was observed [42]. It is possible that GTTR is entering the basolateral (perilymph facing) surface of the outer hair cells by endocytosis and is intracellularly localized to the stereocilia transduction channel.

FM 1-43 FX is a fixable analogue of FM 1-43, which is a styryl dye used to study endocytotic vesicle recycling. Cochlear hair cells exhibit a high rate of endocytosis, and readily accumulate FM 1-43 when it is presented to either their apical and basolateral surfaces [43]. Studies have also shown that FM 1-43 can also penetrate the stereocilia transduction channels, and is retained within the hair cells for at least 14 days [44]. However, accumulation of FM 1-43 or FM 1-43 FX in hair cells after delivery to the scala tympani compartment of an intact cochlea in vivo has not been reported. FM 1-43 FX delivered to the scala tympani is not expected to have direct access to the apical surfaces of the hair cells, which are facing in the scala media, due to the tight-junction network that seals the scala media and also the endocochlear potential that opposes entry of positively charged substances. Therefore, the accumulation of FM 1-43 FX that we observe is likely mediated by endocytosis at the basolateral (scala tympani facing) surface of the hair cells.

In quantifying the distribution of FM 1-43 FX fluorescence intensity in the rows of IHCs and OHCs in tissues fixed three hours after cochlear infusion, we observed a strong base-apex gradient with peak fluorescence near the site of injection in the basal region of the cochlea.
Comparing the distributions between tracers, it is clear that FITC-dextran and Qtracker™-655 fluorescence is concentrated very close to the base (0-2mm) whereas GTTR and FM 1-43 FX fluorescence is concentrated closer to the injection site (2-10 mm for GTTR and 3-6 mm for FM 1-43 FX). The discrepancy here may be due to the increased density of the FITC-dextran solution and Qtracker solutions, due the combination of the substantially higher molecular weight coupled with the larger concentrations injected for these substances (concentrations used were established based on literature reports of labelling protocols for each substance). Salt has demonstrated that the influence of the higher density of FITC-dextran solution, compared to perilymph, is that the solution sinks towards the cochlear base if the animal is injected while the operated ear is facing upward, as performed in our experiments. We suggest that density could be the key factor in interpreting the more basal distribution of FITC-dextran and Qtracker™-655. Interestingly, the method of quantifying punctate labelling in the outer hair cells for GTTR reveals a more similar distribution to the FM 1-43 FX hair cell distribution compared with the GTTR distributions by the trace method. This suggests that the outer hair cell sequestration site at the apical surface may function similarly to the FM 1-43 FX uptake in the hair cells.
4.5 Conclusion

This study provides a preliminary evaluation of a small array of fluorescent substances as drug surrogates to measure distribution after intracochlear drug delivery. Systematic quantification of fluorescence intensity distribution along the spiral axis in whole mount sections was performed for each substance tested. At the three-hour timepoint after intracochlear administration, each of the tracers was localized to the target tissue, as detected by fluorescence microscopy on the fixed whole mount sections. For all the tracers, fluorescence was largely limited to the basal half of the organ of Corti, as expected by the diffusion limitation for transport to the apex. We note that the data presented here represent exploratory studies into the feasibility of using fluorescence probes as a measure of pharmacokinetics in the cochlea, and therefore conclusions regarding the temporal labelling offered by these tracers or the variability between replicates may not yet be drawn. Unique patterns of fluorescence were observed for each of the tracers, with GTTR and FM 1-43 FX appearing as promising sensory-cell specific labels. The present work indicates that fluorescence may serve as a means to infer drug distribution and for the development of ICDD techniques. Further investigation will entail dose-dependent fluorescence patterns, temporal dynamics of the staining patterns observed here, and description of the variability in fluorescence levels across replicates.
References


42. Zhang, Y., et al., Comparison of gentamicin distribution in the inner ear following administration via the endolymphatic sac or round window. Laryngoscope, 2010. 120(10): p. 2054-60.


Advances in microelectromechanical systems (MEMS) technologies are enhancing the development of intracochlear delivery devices for the treatment of hearing loss with emerging pharmacological therapies. Direct intracochlear delivery addresses the limitations of systemic and intratympanic delivery. However, optimization of delivery parameters for these devices requires pharmacokinetic assessment of the spatiotemporal drug distribution inside the cochlea. Robust methods of measuring drug concentration in the perilymph have been developed, but lack spatial resolution along the tonotopic axis or require complex physiological measurements. Here we describe an approach for quantifying distribution of fluorescent drug-surrogate probe along the cochlea’s sensory epithelium with high spatial resolution enabled by confocal fluorescence imaging. Fluorescence from FM 1-43 FX, a fixable endocytosis marker, was quantified using confocal fluorescence imaging of whole mount sections of the organ of Corti from cochleae resected and fixed at several time points after intracochlear delivery. Intracochlear delivery of FM 1-43 FX near the base of the cochlea produces a base-apex gradient of fluorescence in the row of inner hair cells after 1 hour post-delivery that is consistent with diffusion-limited transport along the scala tympani. By three hours post-delivery there is approximately an order of magnitude decrease in peak average fluorescence intensity, suggesting FM 1-43 FX clearance from both the perilymph and inner hair cells. The increase in fluorescence intensity at 72 hours post-delivery compared to 3 hours post-delivery may implicate a potential radial transport pathway into the scala media.
5.1 Introduction

The translation of emerging therapies for sensorineural hearing loss depends on safe and efficacious drug delivery methods and accurate pharmacokinetic assessment of drug distribution in the cochlea [2, 3]. To achieve effective therapy, drugs must be delivered to the fluid compartments of the cochlea and distributed longitudinally along the cochlea’s tonotopic axis within the therapeutic window. Most of the targets for emerging drugs to treat hearing loss are contained within the organ of Corti, including the inner and outer sensory hair cells and the spiral ganglion neurons, which are tonotopically organized along the basilar membrane. For example, intracochlear drug delivery implants that leverage advancements in microelectromechanical systems (MEMS) could be used with cochlear prosthetic devices to provide chronic delivery of pharmacological agents that promote survival of the neuronal cells that are stimulated by the implant [4-6]. Therefore, in evaluating new intracochlear delivery techniques, the spatiotemporal distribution of administered substances should be measured near or inside of the cochlea’s sensory epithelium.

Our group has used electrophysiological measurements to characterize intracochlear delivery of glutamatergic compounds that alter synaptic transmission [7, 8]. DNQX (6,7-dinitroquinoxaline-2,3-dione) is an AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor antagonist that blocks synaptic transmission, and increases threshold for the compound action potential (CAP) [9]. Threshold elevations are measured at discrete frequencies, which correspond to distances along the spiral axis given by the Greenwood function [10]. This provides an assessment of the distribution of DNQX concentration at the inner hair cell synapse along the cochlear axis. DNQX binds the AMPA receptor with a very high rate constant, so the CAP threshold elevations reflect the instantaneous local drug level in the immediate vicinity of the
synaptic terminals. One of the limitations is that the physiological measurement only detects a narrow part of the dynamic range of the total drug-receptor binding events and, therefore, a narrow range of DNQX concentration. Another limitation with the electrophysiological measurement approach is that it is difficult to measure responses in the low frequency regions near the apex of the cochlea. Additionally, our group has previously observed fluid and the formation of granular material in the middle ear, within several days of initial intracochlear surgery, which impact the electrophysiological threshold measurements for chronic timepoints [11].

In addition to measuring the instantaneous drug distribution, for example with DNQX, it would also be advantageous to simultaneously measure several antecedent distributions. This could be achieved, for example, by measuring the effects of compounds with longer receptor binding time constants and unique quantitative readouts. For example, Kang et al have previously used fluorescently-labelled bisphosphonates, which are potential therapeutics for otosclerosis that binds with very high affinity to bone, to assess intracochlear distribution for local and systemic delivery methods [12, 13]. Due to the high affinity binding, the fluorescence distribution essentially reflects the cumulative distribution of bisphosphonate. This technique, however, is limited in spatial resolution, because the cochlea must be manipulated without decalcification to preserve labelling in the bone; drilling to the midmodiolar plane reveals the labelling in the bone at every half turn along the spiral axis.

Fluorescence is measurable over a very wide dynamic range and can be imaged with subcellular spatial resolution on standard epifluorescence and confocal microscopes. Also, a range of excitation/emission spectra exist for various dyes and fluorophores, that can be independently measured on separate optical channels. If several unique fluorescent compounds with varying
affinities for structures within the sensory epithelium were identified, then injecting them together could provide a time-integrated, high-resolution spatiotemporal measurement.

As an early step in developing fluorescent probes for pharmacokinetics, we investigated FM 1-43 FX, a fluorescent marker of endocytosis that can be semi quantitatively measured in the inner hair cells along the cochlear axis. FM 1-43 FX is a fixable version of FM 1-43, a styryl dye that binds vesicle membrane components and is internalized by endocytosis at both the apical and basolateral poles of cochlear hair cells [14]. Endocytosed FM 1-43 is sequestered in the synaptic vesicle pools and subsequently cleared from the cell by vesicle release. Thus, the FM 1-43 serves as a transient hair cell label with a clearance rate that can be predicted from the literature [14]. FM 1-43 also permeates several nonselective ion channels associated with sensory cells, including the stereocilia mechano-transduction channels of cochlear hair cells and the TRPV1 and P2X ion channels on sensory neurons; unbound FM 1-43 that enters through the ion channels stains the intracellular compartments and persists for at least several weeks [15]. Therefore, two different time constants for FM 1-43 retention in hair cells are possible, depending on its mechanism of entry. We hypothesized that FM 1-43 FX, infused into the base of the scala tympani, would transiently accumulate in the hair cells by endocytotic turnover at the basolateral pole. This would result in transient IHC fluorescence, with a time constant dependent on the rate of endocytosis and spontaneous vesicle release in the absence of acoustic stimulation.

We report the distribution of FM 1-43 FX in fixed whole mount sections of the guinea pig organ of Corti at 1 hour, 3 hours, and 72 hours after intracochlear delivery to the scala tympani. Whole mount sections of the guinea pig organ of Corti were fixed, dissected, and analyzed with confocal microscopy.
**Figure 5.1 Method of FM 1-43 FX fluorescence quantification in cochlear sensory epithelium**

a) Intracochlear delivery of FM 1-43 FX in the basal turn of the guinea pig scala tympani using Teflon cannula inserted through cochleostomy and threaded to a total distance of 5 mm from base of the organ of Corti. b) FM 1-43 FX enters sensory hair cells from the perilymph by vesicle recycling at the basolateral surface c) Fixation by intracardial perfusion traps FM 1-43 FX in the hair cells d) Delivery schedule with allotted time for intracochlear transport of FM 1-43 FX in vivo. e) Confocal microscopy image acquisition along the sensory epithelium in whole mount sections of the organ of Corti from base to apex. f) Quantification
of fluorescence intensity as a function of distance from the base of the organ of Corti.

5.2 Materials and methods

5.2.1 Animals

Male Hartley albino guinea pigs (Charles River Laboratories, Inc., Wilmington, MA) weighing 300-500 g were used in this study. All procedures were approved by the IACUC of the Massachusetts Eye and Ear Infirmary. Animals were either anesthetized by isoflurane (1.5%, gas) or a combination of sodium pentobarbital (Nembutal; 25 mg/kg I.P.), fentanyl (0.2 mg/kg I.P.), and droperidol (10 mg/kg I.P.). Anesthesia was administered prior to surgery, and ¼ dose boosters were administered as need to maintain adequate depth of anesthesia.

5.2.2 Surgery and intracochlear drug delivery of FM 1-43 FX

The surgical area was shaved and prepared using sterile technique. Ophthalmic ointment was applied to keep the eyes moist. Atropine (0.04 mg/Kg, S.C.) was administered to control bronchial secretion during surgery. During anesthesia, heart rate, rectal temperature, and blood oxygen levels were monitored every fifteen minutes. For intracochlear drug delivery, the bulla was opened with a blade to visualize the cochlear basal turn and the round window membrane. A cochleostomy was drilled approximately 0.5 mm distal to the round window membrane using a fine drill bit to generate a cochleostomy opening slightly larger than the cannula diameter (OD=201 μm). FM 1-43 FX (FM® 1-43 FX *fixable analog of FM® 1-43 membrane stain, Thermo Fisher Scientific) was prepared at a concentration of 35 μM in artificial perilymph, [(mM) NaCl, 120; KCl, 3.5;
CaCl₂, 1.5; glucose, 5.5; HEPES, 20; NaOH added to bring pH to 7.5]. Drug was loaded in a Hamilton Gastight 1700 Series Syringe with Luer lock and drug solutions delivered with a Harvard Apparatus PHD Syringe Pump. A drug-filled 15 cm length of poly(etherether ketone) (PEEK; Idex Corporation) tubing was used to connect the syringe to a short (4.5 cm) length of Teflon cannula (PTFE tubing with a 101μm ID, 201 μm OD) for intracochlear administration. A small silicone bleb was deposited on the exterior of the cannula, approximately 3 mm from the tip, to control the insertion depth. FM 1-43 FX was infused for two minutes, at a rate of 1 µL/min. Following delivery, the cochleostomy was sealed with carboxylate cement (Durelon™). At least three cochleae were injected for each time point, and both ears of each animal were injected. For each animal, the first injection was performed on the left cochlea at either 3 or 72 hours prior to sacrifice and the contralateral (right) cochlea was subsequently injected either 1 or 3 hours prior to sacrifice, respectively. In some animals the right ear was not injected but collected as a control. For the chronic (72 hr experiments), animals recovered from anesthesia, were returned to their cages, and then re-anesthetized for the contralateral injection 3 hours prior to sacrifice. The levels of noise exposure in the animal care facility were presumed to be lower than that during surgery. In the acute experiments, both ears were injected during a single surgery at 1 and 3 hours prior to sacrifice. Animals were euthanized by pentobarbital overdose (Fatal-Plus) and fixed by intracardial perfusion of a 4% formalin solution.

5.2.3 Tissue Preparation and fluorescence quantification of FM1-43 FX in the organ of Corti

To quantify the distribution of FM1-43 FX fluorescence in the cochlea after intracochlear delivery of FM-143 FX, whole mount surface preparations of the organs of Corti were prepared. After cardiac perfusion with 4% paraformaldehyde, cochleae were dissected from the temporal bones,
post-fixed overnight in the same fixative at room temperature and decalcified in 0.12 M EDTA for 1-2 weeks. After washing with 0.1M PBS, the organ of Corti of each cochlea was dissected under microscope into 12-14 pieces, and mounted on a glass slide for confocal microscopy. Briefly, the decalcified cochlea was bisected along a mid-modiolar plane using a double-edged blade. The tissue partition containing the organ of Corti was removed for each half turn, and the spiral ligament and tectorial membrane were removed. The sections were mounted on glass slides with VECTASHIELD® antifade mounting medium.

A Zeiss LSM 710 laser scanning confocal microscope with a 20X objective was used to image the organ of Corti in adjacent optical sections, each 2.6 µm in thickness. Since we removed the tectorial membrane during the dissection, the hair cells were directly beneath the coverslip allowing imaging of the hair cells without other tissue obstruction. Sodium fluorescein solutions were used to generate calibration curves at several photomultiplier tube (PMT) gain settings. The linear range for each master gain setting was fit to a linear curve by the least-squares error method. However, fluorescence was not converted to concentrations because of the potential for bleeding between sections that would artificially elevate the computed concentrations. Confocal images were analyzed using the ImageJ software (NIH). Seven adjacent optical slices spanning the inner hair cells from the z-stack were combined by sum-intensity projection (Figure 5.2). A 25 µm-thick trace was drawn over the row of inner hair cells in the sum intensity projection. This is effectively the same as a rectangular 3-D trace of the inner hair cells with cross-sectional dimensions of 18.2 µm x 25 µm. The average pixel intensity for the width as a function of distance along the trace, from the base of the organ of Corti, is calculated. The raw signal was processed by averaging the intensity trace into 0.5 mm bins. Binned data were averaged across replicates.
5.3 Results

5.3.1 FM 1-43 FX delivered to the scala tympani permeates cells within the organ of Corti

To examine the delivery of FM-143 FX to the sensory epithelium, we delivered 2 µL of FM 1-43 FX (35 µM) into the scala tympani from a cannula inserted through a cochleostomy and threaded apically to a location approximately 5 mm from the base of the organ of Corti. One hour after intracochlear delivery, fluorescence labeling was observed in the organ of Corti region within approximately the first 10 mm from the base. The cell populations that were strongly labelled include the inner and outer hair cells and the spiral ganglia (Figure 5.3). The inner hair cells were
strongly labelled within the cytoplasm, but the nuclei were not labelled. It appears that only FM 1-43 FX sequestered inside the hair cells is conserved during the fixation process.

**Figure 5.3 FM 1-43 FX labelling at 1 hour and 72 hours after intracochlear delivery in fixed sections from the first turn of the cochlea.**

First row, cytoplasmic staining of inner hair cells with unstained nuclei. Second row, outer hair cells show membrane staining at one hour and increase cytoplasmic staining at 72 hours post-delivery. Third row, cytoplasmic staining of spiral ganglion neuron bodies, higher staining level observed at 1 hour compared to 72 hours post-delivery.
5.3.2 Quantification of longitudinal distribution of FM 1-43 FX fluorescence in the row of inner hair cells

The distribution of FM 1-43 FX in the row of inner hair cells was determined by measuring the sum intensity projection of optical sections spanning the depth of the inner hair cells within the 25 μm-wide rectangular trace (Figure 5.4, a). The contrast between the brightly labelled IHC cytosol and the unstained nuclei and regions between the hair cells generates an oscillation in the intensity of the raw traces. Figure 5.4, b shows an example of the raw intensity distribution in one of the cochleae fixed one hour after injection. Autofluorescence, measured in three control cochleae, was relatively constant throughout the length of the organ of Corti.

Figure 5.4 Longitudinal quantification of IHC fluorescence.

a) Inverted sum intensity projection of optical slices spanning the inner hair cells (the region indicated in yellow) is traced by a line scan in ImageJ (NIH). b) Raw signal of fluorescence intensity for entire length of organ of Corti in sum intensity projection along cochlear axis.
FM 1-43 FX distribution along the row of inner hair cells in cochleae fixed 1 hour after injection was unimodal with a peak at the site of cannula insertion (Figure 5.5). Apical to the injection site, fluorescence decreased to the level of autofluorescence approximately 10 mm from the base. Fluorescence also decreased from the injection site towards the base, in the hook region of the organ of Corti, although levels were higher than autofluorescence. We observed a slight gradient in autofluorescence from base to apex within the row of inner hair cells, measured in the control, with higher-average signals and variability near the base (Figure 5.6).

Figure 5.5 Distribution of FM 1-43 fluorescence intensity in the row of inner hair cells 1 hour after delivery.

Data represents the mean binned intensity at 0.5 mm increments along the row of inner hair cells (n=3). Arrow indicates site of cannula, which is inserted through a cochleostomy and threaded apically to approximately 5 mm from the base of the organ of Corti.
5.3.3 Temporal dependence of fluorescence signal

The strongest signal intensity was observed 1 hour after delivery, with a clear peak at the site of injection (Figure 5.6). We observed a substantial decrease in signal intensity three hours after delivery. Fluorescence intensity decreased over an order of magnitude between 1 hour and 3 hours. Peak fluorescence levels at 72 hours were significantly higher than at 3 hours. Importantly, the distribution of fluorescence along the tonotopic axis was qualitatively similar to that at 1 hour and 3 hours, showing peak levels near the site of injection, with a strong injection-site to apex gradient. For all timepoints tested (1, 3, or 72 hours) the spatial distribution of fluorescence intensity along the tonotopic axis was qualitatively similar, with peak intensity near the site of cannula insertion, and with a decrease in signal intensity in both directions away from the injection location.
Figure 5.6 Temporal dependence of FM 1-43 fluorescence intensity distribution in the row of inner hair cells.

Data represents the mean binned intensity at 0.5 mm increments along the row of inner hair cells (n=3 for 1, 3, and 72 hours).

In two of the 72-hour time point specimens, substantial hair cell loss in the base region only was observed, interspersed by strongly labelled cells (Figure 5.7, left). For these specimens, regions missing hair cells were excluded from the data set. Hair cell loss was only observed near the base of the cochlea (Figure 5.7, right), which was also where the highest levels of staining were observed.
Figure 5.7 Hair cell death in several 72-hour specimens.

Left, example of hair cell loss in the first turn of the cochlea, near the base. Right, distribution of hair cell loss 72 hours after intracochlear delivery of FM 1-43 FX averaged across 5 cochleae.

5.4 Discussion

The purpose of this study was to evaluate the utility of a fixable analogue of FM 1-43 FX as a surrogate drug compound to semi-quantitatively measure drug distribution for intracochlear drug delivery into the scala tympani. For example, using other pharmacokinetic techniques it has been shown that a single intracochlear injection at the base of the cochlea is not effective in achieving drug distribution throughout the scala tympani, especially towards the apex [13, 16, 17]. Ideally, a pharmacokinetic analysis “toolkit” would comprise a small library of compounds with unique retention times and quantitative readouts that could be co-administered to provide temporal as well as spatial information. This study represents one potential element of that toolkit; quantitatively
evaluating the distribution of FM 1-43 FX in the row of inner hair cells at time points 1, 3, and 72 hours after delivery.

The most direct route for FM 1-43 FX entry into the hair cells from the scala tympani is through endocytosis at the basolateral cell surface. While FM 1-43 can, in principle, enter virtually any cell type by endocytosis, cells with a high rate of vesicle recycling accumulate FM 1-43 FX more efficiently [18]. Cochlear inner hair cells have a high rate of endocytotic recycling at their basolateral pole, where they synapse the spiral ganglion neurons. Even in the absence of acoustic stimulation, IHCs exhibit a high rate of spontaneous vesicle release [19]. In addition to entry by basolateral endocytosis, hair cells are known to have an especially high affinity for FM-143 when it is presented at their apical (endolymphatic) surface, due to both a high rate of apical endocytotic recycling, and facile entry through the stereocilia transduction channel [14, 15].

We observed strong fluorescence in the row of inner hair cells at 1 hour after FM 1-43 FX delivery to the scala tympani, with peak intensity near the intracochlear delivery. The gradient of fluorescence intensity along the cochlear partition (Figure 5.5) at this 1-hour timepoint is consistent with diffusion-limited transport along the length of the scala tympani. In agreement with experimental results by other methods, we observed a steep gradient in fluorescence intensity from the site of injection that decreases towards the apex [16, 20]. Fluorescence also decreased from the injection site toward the base of the organ of Corti, which is similar to the observations our group has previously found in electrophysiological assays with threshold elevations after DNQX delivery [17]. Due to the presence of the efflux route through the cochlear aqueduct in guinea pigs, flow driven by the syringe pump is expected to proceed from the cannula tip towards the base of the scala tympani during the 2-minute injection period. The pressure generated at the cannula exit by the pump exceeds the positive cerebrospinal fluid (CSF) pressure at the cochlear aqueduct, such
that the pressure gradient results in flow toward the base. Once the pump is stopped, drug near the cochlear aqueduct may be diluted by physiological mixing of perilymph and CSF [21]. Furthermore, diffusional exchange between the scala tympani and the cerebrospinal fluid in the cochlea aqueduct is expected near the cochlear base. Furthermore, due to CSF pressure, a slow, basal-to-apical directed flow may occur once the pump is stopped, if there are any large efflux routes in the system, such as the cochleostomy site. These phenomena may explain the decrease in fluorescence intensity from the injection site to the base. Fluorescence seen in inner hair cells 1 hour after scala tympani infusion may be a result of FM 1-43 FX accumulation by endocytosis at the basolateral surface facing the perilymph, the obvious uptake site after injection into the scala tympani. In a similar experiment, Leake and Synder injected horseradish peroxidase into the scala tympani, and 1 hour after delivery, HRP reaction product in the hair cells was observed only in the infranuclear regions, near the basolateral surface [22]. They also noted that HRP did not penetrate the reticular lamina, and was not present anywhere in the endolymphatic space.

The exchange of FM 1-43 FX between the hair cells and the perilymph is a dynamic process that results from vesicle recycling: FM 1-43 FX is internalized by endocytosis, retained in the vesicle pools, and released by vesicle fusion. The amount of FM 1-43 FX that accumulates inside the inner hair cells, therefore, is dependent on the concentration in the perilymph. Once FM 1-43 FX is cleared from the perilymph there will be no source of FM 1-43 FX at the basolateral pole of the hair cells; therefore, we expected that fluorescence intensity inside the hair cells will decrease as FM 1-43 FX is cleared from the perilymph.

Once internalized in the hair cells by endocytosis, FM 1-43 mostly remains bound to the vesicle components and sequestered in the vesicle pools, and is subsequently cleared from the hair cell by vesicle release [18]. When applied to the basolateral surface of inner hair cells ex vivo, the half-
life of fluorescence signal within the hair cell (FM 1-43 retention) is approximately 120 seconds [14]. We observed a marked decrease in FM1-43 FX labeling in tissues examined 3 hours after infusion into the cochlea relative to the 1-hour time point. Although the two interacting time constants for clearance from the hair cells and clearance from the perilymph cannot be separately determined from this measurement, the data suggest that clearance from the perilymph is the primary determinant in the kinetics of FM 1-43 FX in the IHCs. We observed a qualitatively similar longitudinal gradient at 1 and 3 hours (Figure 5.6), which suggests that FM 1-43 FX is removed from the perilymph by a radial elimination, for example into adjacent tissue or fluid compartments, rather than longitudinal clearance which would be expected to give a different distribution. Salt et al. measured the clearance rate of an extracellular cationic compound, TMPA (trimethylpropylammonium), injected into the basal turn of the scala tympani using a TMPA-selective electrode, measuring nearly an order of magnitude decrease in TMPA concentration within 90 minutes after intracochlear delivery [23]. Here again, the kinetics are governed by individual time constants for the rate of FM 1-43 FX internalization into the hair cells, clearance from the hair cells, and clearance from the scala tympani. Measuring the changes in fluorescence levels between the 3 hour and 72 hour time points represents another opportunity to probe the kinetics of uptake and clearance. In this study, fluorescence levels in the IHCs rose from 3 and 72 hours after delivery (Figure 5.6). Although we did not directly measure interscalae communication, we speculate that one potential mechanism explaining this increase is that FM 1-43 FX may have, over a prolonged time, gained access to another uptake site on the hair cells, perhaps even after clearing from the perilymph. One candidate site for uptake is the apical surface of the hair cells, facing the scala media. However, access to apical pole of the hair cells would first require FM 1-43 FX entry into the endolymph, which is
impeded by the tight junction network that separates the scala tympani and scala media and the
electrochemical gradient across this cellular network that opposes the positively charged FM 1-43
FX molecule [24, 25]. The distribution of labeling at 72 hours demonstrated the same longitudinal
gradient we observed in acute (1 and 3h) experiments, with a strong peak in intensity near the
injection site. This runs counter to the possibility that the FM 1-43 FX is transported into the
systemic circulation and then back to the scala media, where a more uniform intensity distribution
along the cochlear axis might be expected. Hair cells are known to have a high affinity for FM 1-
43 at their apical surface by entry of unbound FM 1-43 through the stereocilia transduction
channels, and a high rate of apical endocytosis. In contrast to transient labelling at the synapse,
where the FM 1-43 FX may be sequestered in, and quickly released from the vesicle pools, FM 1-
43 FX entry at the apical surface in sensory cells can lead to labeling of the cellular compartments
that is persistent for at least 14 days [15]. Therefore, if some of the FM 1-43 FX enters the scala
media through local pathways, such as through the modiolus, the spiral ligament, or cochlear
partition, it may permeate the hair cells more efficiently, even at low concentrations.

We observed substantial hair cell loss near the base of the cochlea in two of the 72-hour
experiments that was not observed in any of the acute experiments, or in 72-hour time points after
injections with other dyes. While potential other sources of damage cannot be ruled out, the fixable
analogue of FM 1-43, (FM 1-43 FX), used in this study was shown to have some toxicity to the
sensory epithelium ex vivo [26]. That the distribution of hair cell loss occurs near the base (Figure
5.7), coincident with levels of staining, may implicate toxicity of FM 1-43 FX at the concentration
used. This study represents an innovative approach to measuring the spread of drugs through the
cochlea after intracochlear injection. In these experiments, drug was infused at the base of the scala
tympani over a two-minute period, and measured at discrete time intervals (1, 3 and 72 hours after
delivery. However, many new technologies that are being developed for intracochlear delivery are aimed at delivering drugs in a chronic fashion over, perhaps, weeks or months. The present results are applicable to delivery that has occurred shortly before the measurement timepoint, for example, within 72 hours. However, further studies should be conducted to determine if the fluorescence we observe at 72 hours persists for longer time periods, and therefore, that the fluorescence levels at chronic timepoints reflect the cumulative distribution within the cochlea.

5.5 Conclusions

Here, we describe the use of a hair cell permeant fluorophore, FM 1-43 FX, as a surrogate for small molecule drug substances, to quantify the spatial drug distribution in the sensory epithelium after infusion into the scala tympani. We demonstrate quantification of the complete spatial distribution in the inner hair cells after intracochlear delivery. Using confocal microscopy, we were able to measure the entire distribution of fluorescence along the cochlear axis in fixed whole mount sections of the organ of Corti at the subcellular level. Individual hair cells are recognized in the signal by an oscillation that corresponds to the contrast between the brightly labelled cytosol and the unlabeled nuclei and space between cells. By measuring the distribution of intensities at 1, 3 and 72 hours, we observed a paradoxical increase in fluorescence between 72 hours, which we speculate was due to FM 1-43 FX entering the scala media. Further investigation is warranted to elucidate this putative phenomenon, which may have important implications for the potential transport routes of drugs with similar physicochemical properties to FM 1-43 FX. At acute timepoints, at least until 3 hours after delivery, FM 1-43 FX appears to be a transient label that is taken up by the known endocytotic recycling route and cleared from the hair cells by vesicle release. The fluorescence distribution during this time is expected to be a function of the perilymph concentration. The observation that the fluorescence signal increases between 3 and 72 hours, may
be explained by a mechanism in which some tracer FM 1-43 FX may be reaching the endolymph and entering the hair cells at their apical surfaces, where it is internalized more efficiently and at lower concentrations. The distribution of fluorescence at 72 hours suggests that FM 1-43 FX may be capable of entering the scala media, where it would have direct access to the apical surfaces of the hair cells. If so, the fact that the distribution of fluorescence at this chronic timepoint reflects the initial post-injection distribution of FM 1-43 FX in the perilymph would correspond to a radial transport route, such as diffusion through the adjacent scalae.
References


Mixed Reversible Covalent Crosslink Kinetics Enable Precise, Hierarchical Mechanical Tuning of Hydrogel Networks

This work has previously been published by AM Ayoob, VY Yesilyurt et al.[1]

6.1 Introduction

Hydrogels from polymeric networks play a central role in a number of medical applications, including as cellular scaffoldings, drug delivery systems, soft tissue replacements, and wound dressings.[2-4] Precise engineering of hydrogel networks to produce mechanically defined biomaterials may improve their capacity to recapitulate or mimic the functional dynamics of native tissues.[5] In particular, the mechanical behavior of hydrogels under dynamic strain loading regimes are important factors influencing performance in cell scaffolding and as tissue replacement applications, and must be tailored to suit specific tissues.[6-9] For example, stress relaxation in the extracellular matrix (ECM) is a key attribute of cell-ECM interactions and an important design parameter for hydrogels for tissue engineering.[10]

A variety of parameters control the physical properties and biological interactions of hydrogels materials, and may ultimately dictate its success or failure.[11] Reversible chemical interactions have emerged as a useful crosslinking strategy to construct mechanically dynamic shear-thinning and self-healing hydrogels. A variety of transiently crosslinked systems have been demonstrated including those based on hydrogen bonding and electrostatic interactions, as well as host-guest and receptor-ligand pairs.[12-14] Due to the reconfigurable nature of these interactions, dynamically crosslinked systems can exhibit stress relaxation and self-healing behavior. Those with shear-thinning behavior can be readily injected through high gauge medical syringes.[15]
However, these systems have typically been designed from repeating weak interactions along the polymer backbone, which limits their mechanical performance. It is difficult to engineer mechanical properties in a precise fashion a priori and modifications inherently alter other properties of the network such as biochemical characteristics or effective mesh size. Recently, multiple metal coordination crosslinks with distinct kinetic rates were utilized to introduce multiple relaxation modes in viscoelastic hydrogel networks. This was a demonstration of decoupling the dynamic mechanical behavior from the backbone structure of a hydrogel network.[16] This orthogonal, hierarchical approach offers a promising strategy for the rational design of soft matter with multiple relaxation modes. Dynamic covalent crosslinks have also been explored to produce structurally dynamic polymers and polymer networks, and control relaxation timescales independent of other structural network attributes.[17, 18] However, the a priori engineering of hydrogel networks with precise relaxation timescales, independent of the repeating polymer structure, remains a challenge. Herein, we describe a systematic approach to tuning the relaxation time in viscoelastic polymer networks using reversible covalent network linkages.

The reversible covalent complexation of phenylboronic acid (PBA) with cis-1,2 or cis-1,3 diol compounds has been investigated as a dynamic link to produce supramolecular structures and hydrogels.[19, 20] Recently, it was reported that shear thinning and self-healing networks assembled from various phenylboronic acid and glucose-like diols can be developed for pH-sensitive delivery of therapeutic macromolecules.[21] Interestingly, in these studies, the location of the crossover frequency during dynamic rheology was sensitive to the pKa of the PBA used. We hypothesize that precise, orthogonal tuning along a continuum of dissipation timescales can be achieved by forming networks containing multiple, kinetically distinct, PBA-diol pairs. Herein, we develop a methodology to precisely tune the mechanical properties of PEG networks formed
from mixtures of two different PBA derivates with unique diol complexation rates, 4-carboxyphenylboronic acid (PBA) and o-aminomethylphenylboronic acid (APBA) (Figure 6.1).

**Figure 6.1 Hydrogels formed from reversible-covalent crosslinks**

a) Schematic of hydrogels from 4-arm PEG macromers crosslinked by b) a mixture of reversible covalent complexes of different phenylboronic acid derivatives with cis-diols. c) Both slow and fast crosslinks behave as dissipation crosslinks for relatively slow strain rates, whereas only the fast dynamic crosslinks can dissipate stress for fast strain rates.
6.2 Materials and Methods

4-Arm PEG-Amines (MW: 5, 10, and 20 kDa) were purchased from JenKem Inc. 4-Carboxyphenylboronic acid, Dichloromethane (DCM), Dimethylformamide (DMF), 2-Formylphenylboronic acid, D-(+)-Gluconic acid δ-lactone, Triethylamine (NEt₃) Sodium borohydride (NaBH₄), were purchased from Sigma-Aldrich. The coupling reagent O-(Benzotriazol-1-yl)-N,N,N,N-tetramethyluroniumhexafluorophosphate (HBTU) was purchased from Chem-Impex Inc., 1-Hydroxybenzotriazole Monohydrate (HOBt) was purchased from TCI Chemicals Inc., Regenerated Cellulose Dialysis tubing was purchased from Spectrum Labs (MWCO: 1 and 3.5 kDa). ¹H NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer using the residual proton resonance of the solvent as the internal standard. Chemical shifts are reported in parts per million (ppm).

6.2.1 Synthesis of Hydrogel Precursors

4-Arm-Polyethyleneglycol NH₂HCl salt (1.0 g, Mw 5,000 g/mol), and 0.18 g 2-Formylphenylboronic acid (1.2 mmol) was dissolved in 10 mL of MeOH, and 0.3 mL of triethylamine was added to reaction mixture. The reaction was allowed to proceed for 72 hr at room temperature under argon gas. Afterwards, the reaction was cooled to 0 °C, and 90 mg of NaBH₄ (2.4 mmol) was added portion wise. The reaction was left to proceed for 12 hr at room temperature. Next, the methanol was removed, and the resultant crude product was dissolved in DI water, and pH was balanced to 7 with 2 N HCl. The mixture was dialyzed against DI water (1,000 MWCO) for 24 hr, and then lyophilized. ¹H (400 MHz, D₂O, δ): 7.1-7.4 (m, 16H), 4.0 (s, 8H), 3.6 (bs, PEG), 3.0 (bs, 8H).
6.2.2 Hydrogel Fabrication

10 w/v% (polymer mass/total volume) aqueous stock solutions of three precursors, PEG-PBA, PEG-APBA and PEG-diol were prepared in either 0.1 M phosphate buffer, pH 7.4, 8.5, or in 0.1 M MES, pH 6. To formulate the hydrogels, appropriate volumes of stock solutions were combined to yield a final total polymer concentration of 10 w/v%. Gelation occurred within 30 seconds.

As an example: 0:100 PBA:APBA gels at pH 7.4: Equal volumes of 10 w/v% stock solutions of APBA and PEG-diol polymers prepared in pH 7.4 buffer were mixed in 1 mL of eppendorf tube to give the gel, consisted of 5 w/v% of APBA and 5 w/v% of PEG-diol polymers (total 10 w/v% of polymer concentration in the gel)
**50:50 PBA:APBA gels at pH 7.4:** Appropriate volumes of 10 w/v% stock solutions of APBA and PBA polymers were taken into 1 mL of eppendorf tube, followed by the addition of appropriate volume of 10 w/v% stock solution of PEG-diol polymer to yield gels, consisted of 2.5 w/v% of APBA and 2.5 w/v% PBA polymers and 5 w/v% of PEG-diol polymer. (total 10 w/v% of polymer concentration in the gel).

The following table shows the weight percentages of 5 kDa polymers in any given particular gel composition after mixing the appropriate volumes of 10 w/v% each stock solution. Similarly, gels from 10 and 20 kDa polymer precursors are composed of the same w/v % as the 5 kDa formulations (below).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>5 kDa PEG-PBA (w/v%)</th>
<th>5 kDa PEG-APBA (w/v%)</th>
<th>5 kDa PEG-diol (w/v%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>PBA:APBA</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>75:25</td>
<td>3.75</td>
<td>1.25</td>
<td>5</td>
</tr>
<tr>
<td>PBA:APBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50:50</td>
<td>2.5</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>PBA:APBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25:75</td>
<td>1.25</td>
<td>3.75</td>
<td>5</td>
</tr>
<tr>
<td>PBA:APBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0:100</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
6.2.3 Dynamic Rheology

Mechanical properties of the hydrogels was studied using TA Instruments AR 2000 rheometer fitted with a Peltier stage set to 37 °C. Oscillatory strain amplitude sweep measurements were conducted at a frequency of 10 rad/s. Oscillatory frequency sweep measurements were conducted at a 1% strain amplitude. All measurements were performed using a 20 mm 4° cone. Step-strain measurements were performed using 10 w/v% of hydrogel formed from 50:50 mixtures of PBA:APBA at pH 7.4 to determine the recovery of hydrogel mechanical properties following network rupture at high strains. For this purpose, 50:50 PBA:APBA gel was subjected to high strain (γ=500%) and the storage modulus (G’) immediately dropped with corresponding inversion of G’ and loss modulus (G”) indicating network disruption. When high strain was discontinued and a low magnitude strain (γ=0.5%) was applied, the hydrogel exhibited 100% recovery of both G’ and G” within a few seconds after strain-induced failure, which was reproducible upon additional strain cycles.
6.2.4 Modeling relaxation spectra

The relaxation spectra for the various PBA:APBA mixtures were determined from rheological frequency sweep data, utilizing MATLAB’s *lsqcurvefit* function. Since two kinetic rate constants were present in the gels, one for each PBA-diol and APBA-diol complexes, a two-mode log-normal distribution was assumed for the distribution of relaxation times:

\[ H(\tau) = A_1 \exp \left( -\frac{(\ln(\tau) - \ln(\tau_1))^2}{2\sigma_1^2} \right) + A_2 \exp \left( -\frac{(\ln(\tau) - \ln(\tau_2))^2}{2\sigma_2^2} \right) \]

This distribution can be obtained by solving for the parameters the sum of squares of the difference of experimental and model G’ and G’’ data. The model data are calculated using:
\[ G'(\omega) = \int_{-\infty}^{\infty} H(\tau) \frac{\omega^2 \tau^2}{1 + \omega^2 \tau^2} d\ln(\tau) \]

\[ G''(\omega) = \int_{-\infty}^{\infty} H(\tau) \frac{\omega \tau}{1 + \omega^2 \tau^2} d\ln(\tau) \]

6.3 Results

6.3.1 Effect of stoichiometry on the relaxation behavior under dynamic shear

4-arm PEG-PBA macromers were produced by either reacting 4-arm PEG-NH\(_2\) (5-20 kDa) with O-(Benzotriazol-1-yl)-N,N,N,N-tetramethyluroniumhexafluorophosphate (HBTU) activated 4-carboxyphenylboronic acid to produce PEG-PBA, or by reductive amination of 4-arm PEG-NH\(_2\) with 2-formylphenylboronic acid to produce PEG-APBA.[21] To produce the 4-arm PEG-diol macromers, D-Glucolactone was reacted with 4-arm PEG-NH\(_2\) in the presence of triethylamine. All gel formulations were produced by dissolving the PEG-PBA and PEG-diol macromers in buffered aqueous solution at 10 w/v\%, and homogenized with vigorous mixing.

Five unique hydrogel formulations were produced by equimolar mixing of PEG-PBA and PEG-diol macromers, while varying the relative ratio of PBA:APBA macromers. For example in a 50:50 PBA:APBA gel, 50% of the phenylboronic acid derivatives are PBA and 50% are APBA. All mixtures exhibited viscoelastic network behavior, evidenced by the intersection of \(G'\) and \(G''\) on frequency-sweep rheometry curves (Figure 6.4, a-e). Owing to dynamic nature of the network crosslinks, the mechanical moduli observed in dynamic rheology are highly dependent upon the timescale of deformation: strain frequencies faster than the crosslink dynamics evoke an elastomeric response, while low frequency deformations allow stress dissipation as network junctions disengage. These characteristics are also true of entangled polymer networks, but the timescales of the mechanical response depend on structural factors such as persistence length and...
Molecular weight, precluding hierarchical design.[22] In order to delineate the dissipation dynamics of the five mixtures, the rheological data were fit to a model of an infinite spectrum of Maxwell elements, and the dissipation timescales were plotted for the various mixtures (Figure 6.4, f).[23] The model assumes an infinite parallel arrangement of Maxwell elements (a compliance (capacitance) and a dashpot (resistance) in series). Each frequency location ($\omega$) is represented as a Maxwell element with time constant $\tau$, where $\tau = 1/\omega$. The model relaxation spectrum, $H(\tau)$, describes the entire distribution of Maxwell elements across timescales. The magnitude of $H(1/\omega)$ at a particular location is proportional to the energy dissipation at frequency $\omega$. The area under the distribution, $\int_{-\infty}^{1/\omega} H(\tau) d\ln(\tau)$, is proportional to the energy storage at frequency $\omega$ and the plateau of $G'$ from the rheology data. Mixtures of PBA and APBA have uniform, unimodal Maxwell distributions similar to their parent pure PBA or APBA gels, but with intermediate dissipation timescales. Strikingly, the dissipation timescale of PBA:APBA mixtures is exquisitely sensitive to the relative concentrations of PBA and APBA (Figure 6.4).

There were no significant differences in the elastic modulus ($G'$ at the crossover frequency) or in the shape of the distribution of Maxwell elements for each of the mixtures, indicating that the accessible mechanical properties for each of the mixtures is the same, but that the time scales required to observe those properties is shifted, which we attribute to the differences in $pK_a$ of the phenyl boronic acids. PBA has a higher reported $pK_a$ than APBA, 7.8 and 6.5-6.7, respectively.[24-26] This is significant because has been reported that the tetrahedral boronate anion is the predominant species that participates in PBA-diol complexation.[27] Therefore, the differences observed in the crossover frequencies corresponding to $pK_a$ differences in phenylboronic derivatives directly connect the molecular kinetics of the reversible crosslinks to the macroscopic dynamic mechanical behavior. Gels formed entirely from PBA-diol interactions...
(100:0 PBA:APBA mixture) exhibit an order of magnitude shorter dissipation timescale compared to those of entirely APBA (0:100 PBA:APBA mixture) (Figure 6.4, f). Consistent with these results were the decreasing exponential decay rates of the stress relaxation modulus for increasing APBA content, measured in the step strain paradigm (Figure 6.5, a). The rate of stress relaxation is an important consideration for self-healing materials, especially in the context of injectable materials or dynamic cell culture systems: the rate of shear must be slower than the rate of the dynamic crosslinks. Previously, it was reported that 100:0 PBA:APBA gels are capable of self-healing, while 0:100 PBA:APBA does not self-heal when exposed to large strain.[21] Here we investigated the ability to self-heal in networks formed from APBA and PBA. We found that while 0:100 PBA:APBA did not exhibit self-healing, 50:50 PBA:APBA did self-heal rapidly after exposure to large strains (Figure 6.5, b).
Figure 6.4 Rheological characterization of PBA:APBA mixtures.

a-e) Oscillatory rheology measurements of PEG hydrogels (10 w/v%) from stoichiometric amounts of 5kDa PEG-PBA and 5 kDa PEG-diol macromers formed at pH 7.4 The relative amounts of PBA to APBA was varied from 0:100 to 100:0 %. Experimental rheology data (triangles) are overlaid with model rheology curves (solid lines). f) Infinite spectra for the mixtures in a-e), from 0:100 PBA:APBA (green trace) to 100:0 PBA:APBA (black trace).
6.3.2 Effect of pH

We further investigated differences in the PBA-diol complexation kinetics between the phenylboronic acid crosslinks by measuring the mixtures’ mechanical properties at various pH levels (Figure 6.6, a-c). The pH is known to affect the balance between the bound and unbound states in PBA-diol complexes, with lower pH favoring the unbound state. The effect of pH is relative to the pKa of the phenylboronic acid, since it is predominately the conjugate base of the phenylboronic acid, the phenylboronate anion, that complexes cis-diols and hydroxy acids.[27] Hydrogels formed from PBA-diol crosslinks are thus sensitive to pH changes, with low pH relative
to the PBA pKa resulting in faster self-healing and gradual flow under gravity.[19] By contrast, gels at high pH relative to the PBA pKa have the characteristics of permanently crosslinked networks with limited ability to deform and self-heal. It was expected that increasing the pH would result in shifts of the dissipation timescales toward slower relaxation. For all PBA:APBA mixtures tested, there was at least an order of magnitude slower dissipation timescale at pH 8.5 compared to pH 7.4. For the 100:0 and 50:50 PBA:APBA mixtures, this temporal shift was accompanied by a two fold increase in modulus (Figure 6.6, a & c). The increase in elastic strength (G’ at plateau, also reflected in the area under the Maxwell distribution curve) with increased pH indicates a higher crosslink density, and is consistent with a higher proportion of bound BA-diol at elevated pH. The temporal shift is interpreted as a high pH induced shift in equilibrium constant favoring the tetrahedral boronate-diol complex and reducing the rate of formation of unbound boronate ion or boronic acid, thus reducing the rate of stress dissipation through crosslink cleavage. Interestingly, for the 0:100 PBA:APBA mixtures, there was a near order of magnitude increase in the dissipation timescale with increased pH, without a proportional increase in elastic strength. We interpret this as a decrease in the reverse complexation rate without a substantial increase in the proportion of bound complexes. This could be due to network heterogenities caused by the observed rapid gelation that occurs at pH values far above the boronic acid pKa. However, 50:50 PBA:APBA gels, there was a significant decrease in the elastic energy stored, and a complete loss of elastic character in the 100:0 PBA:APBA accompanying decreases in pH.
6.3.3 Effect of Molecular Weight

To study the potential of this approach for tuning the dissipation timescales, we examined the effect of the average molecular weight between crosslinks ($M_c$). The $M_c$ in this system is determined by the macromers size, thus we synthesized 5 kDa, 10 kDa, and 20 kDa 4-arm PEG macromers functionalized with PBA, APBA, or cis-like diol. The effect of increasing the $M_c$ is to reduce the number of elastically active crosslinks ($\nu_e$) per unit volume ($\tilde{V}$). For elastomers, the
relationship between shear modulus is directly proportional to the elastically active crosslink density:[28]

\[ G = kT \frac{\nu_e}{\nu} \]  

(1)

Thus, we expected that the gels formed from larger macromers would have a smaller elastic modulus, but that the dissipation timescales would be unaffected. Dynamic rheology of gels from 5 kDa, 10 kDa, and 20 kDa macromers reveals a reduction in ultimate elastic modulus with increasing molecular weight, without a shift in dissipation timescale for a given PBA:APBA mixture (Figure 6.7, a-e). Importantly, the shift in timescales between mixtures was approximately uniform for the three molecular weights tested. These results demonstrate the orthogonality of the approach, showing that the tuning achieved in Figure 6.4, f and Figure 6.5, a can be carried across other aspects of the network architecture.
Figure 6.7 Independence of Relaxation Time Scale and Molecular Weight

a-e) Relaxation spectra for PBA:APBA mixtures at pH=7.4 for three different molecular weights: 5 kDa, 10 kDa, and 20 kDa phenylboronic acid and cis–diol macromers. Relaxation timescales increase with increasing percentage of APBA, but are independent of macromers molecular weight. Increasing moduli are concomitant with decreasing macromers molecular weight, indicating an increase in active crosslink density.
6.4 Discussion

In summary we have demonstrated the utility of mixtures of kinetically unique covalent crosslink dynamics in tuning the time dependence mechanical response of bulk hydrogels. The self-healing nature of these systems makes them amenable for injectable clinical applications. This strategy builds on recent work aimed at hierarchical tuning of network dynamics in transiently crosslinked systems [16]. Important implications of tuning mechanical dynamics in hydrogels includes advanced three-dimensional cell culture environments that more closely mimic native tissues.
References


7 Summary, Continued Work, and Future Directions

The peripheral auditory system is a critical sensory organ that enables communication and enjoyment through music and other auditory stimuli; therefore, hearing impairment, the most common sensory deficit, has profound effects on the millions that suffer sensorineural hearing loss. Currently, many emerging drug candidates are targeted at the sensorineural cells to repair or replace these critical components of the auditory chain that transduce acoustic pressure waves into afferent neural signals. Given the difficulty in localizing and maintaining therapeutic levels of drug substances, effective and viable delivery strategies must first be conceived and demonstrated to facilitate the translation of emerging compounds. Furthermore, effective methods of evaluating pharmacokinetics in animal models must be utilized to test novel delivery approaches before advancement to human use. Therefore, the primary objective of this thesis was to investigate innovative solutions to pharmacokinetic analysis in the cochlea and propose novel materials-based platforms that could be adapted for controlled-release applications in the cochlea.

To achieve these important goals, we first demonstrated a computational model designed and implemented for intracochlear delivery near the base of the scala tympani compartment. Specifically, we compared model results for intracochlear vs. intratympanic delivery, and evaluated the effectiveness of sustained intracochlear drug delivery on improving the distribution of drug concentration along the cochlear axis. We then demonstrated a novel technique of using fluorescent tracer probes, as drug surrogates, to quantify spatial distribution along the cochlea’s sensory epithelium, which contains the therapeutic target cell population for most emerging drugs candidates. Tracers were administered directly into the scala tympani compartment by a controlled microfluidic injection in vivo. By performing confocal microscopy on whole mount sections of the
dissected guinea pig cochlea, we were able to measure the intracochlear distribution with unprecedented spatial resolution. Further, we evaluated the distribution of a lead tracer candidate, FM 1-43 FX, at multiple timepoints to provide additional context of how this tracer can be used in chronic drug delivery experiments. Finally, we presented novel materials-based strategies to design injectable hydrogel materials that can be adapted for use as controlled-release vehicles for auditory applications. However, there are additional steps that need to be taken to advance this work towards the development of novel drug delivery approaches.

7.1 Additional studies on fluorescent tracers

The small array of fluorescent drug-surrogates used in this work were chosen based on their known ability to label hair cells in vitro. FM 1-43 FX, the lead candidate tracer, exhibits a non-monotonic increase in fluorescence between 1 and 72 hours, and it was suggested that FM 1-43 FX enters the endolymphatic space. The studies described here were limited to quantification of fluorescence levels in the whole-mount sections containing the organ of Corti; however valuable information could be obtained about the transport route for FM 1-43 FX by measuring its distribution in other tissue, including the spiral ligament or by collecting the perilymph to examine the FM 1-43 FX concentration as various timepoints. Such additional studies would provide great insight on transport routes in the cochlea, not only for FM 1-43 FX, but also for other cationic small molecule drugs.

Repeating the experiments described here for much longer time horizons would also be warranted if FM 1-43 FX was to be employed as a chronic fluorescent tracer substance. The ability for FM 1-43 FX to be retained in the hair cells over weeks or months would be useful as a tracer for extended delivery paradigms where a cumulative measure of delivery is desired.
The ideal “toolkit” for pharmacokinetic analysis would be to have an array of tracers, each with known time constants for retention in the hair cells or other cell populations preserved in the whole mount preparations. The optical method of measuring fluorescence implemented here lends itself to the orthogonal quantification of multiple fluorophores on separate optical channels. Identification of tracers that are not retained for long time periods could be used with FM 1-43 FX, and other tracers identified here, to provide a more complete temporal “map” in addition to the excellent spatial resolution demonstrated.

### 7.2 Sustained intracochlear delivery

The fluorescent tracer studies performed here were controlled short-duration infusions. Our modeling results demonstrate that a single intracochlear infusion is likely insufficient to maintain intracochlear concentrations for a desired duration or to distribute drug concentration to the apical regions. Therefore, continued experiments could employ a reciprocating delivery paradigm where fluorescent tracers are infused multiple times, without volume change, would confirm or deny our modeling results, that sustained intracochlear delivery dramatically improves the apical distribution of drug. For this, the FM 1-43 FX tracer could be used and would enable the precise spatial resolution that would be required to resolve concentration differences between single and repeated intracochlear injections, along the cochlear axis.

Furthermore, injectable polymer formulations as sustained release vehicles should be investigated that will enable sustained drug concentrations in the base of scala tympani. Here we have designed and mechanically characterized one such system, enabling precise mechanical tuning that can be optimized, using the protocol described here, for injectability through high gauge conduits and to minimize the disruption of the mechanical characteristics of the cochlear fluid compartments. This would represent a first-in-class injectable system, that can be placed directly in the cochlear fluids.
with minimal mechanical disruption of the normal cochlear mechanics. The interactions between drug solute molecules and the polymer matrix should be optimized to enable maintenance of local concentrations in the basal region of the cochlea. This could be achieved by tethering drugs to boronic acid, diol or glucose derivatives, such that the drug solutes are retained and slowly released from the gel network. Alternatively, non-covalent interactions between hydrophobic solutes and hydrophobic domains in the gel could be employed to slow the rate of release and increase drug solubility and loading in the gel.