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Persistence, Distribution, and Impact of Distinctly Segmented Microparticles on Cochlear Health following *In Vivo* Infusion^{3*}

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Abstract

Delivery of pharmaceuticals to the cochleae of patients with auditory dysfunction could potentially have many benefits from enhancing auditory nerve survival to protecting remaining sensory cells and their neuronal connections. Treatment would require platforms to enable drug delivery directly to the cochlea and increase the potential efficacy of intervention. Cochlear implant recipients are a specific patient subset that could benefit from local drug delivery as more candidates have residual hearing; and since residual hearing directly contributes to post-implantation hearing outcomes, it requires protection from implant insertion-induced trauma. This study assessed the feasibility of utilizing microparticles for drug delivery into cochlear fluids, testing persistence, distribution, biocompatibility, and drug release characteristics. To allow for delivery of multiple therapeutics, particles were composed of two distinct compartments; one containing polylactide-co-glycolide (PLGA), and one composed of acetal-modified dextran and PLGA. Following *in vivo* infusion, image analysis revealed microparticle persistence in the cochlea for at least 7 days post-infusion, primarily in the first and second turns. The majority of subjects maintained or had only slight elevation in auditory brainstem response thresholds at 7 days post-infusion compared to pre-infusion baselines. There was only minor to limited loss of cochlear hair cells and negligible immune response based on CD45+ immunolabeling. When Piribedil-loaded microparticles were infused, Piribedil was detectable within the cochlear fluids at 7 days post-infusion. These results indicate that segmented microparticles are relatively inert, can persist, release their contents, and be functionally and biologically compatible with cochlear function and therefore are promising vehicles for cochlear drug delivery.

Keywords

segmented microparticle; electrohydrodynamic co-jetting; cochlear drug delivery; biocompatibility

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1. Introduction

There is increasing need for local drug delivery to the cochleae of patients with various cochlear pathologies, including cochlear implants. Local drug delivery could be used to increase survival and function of remaining auditory neurons following trauma such as implantation^{1,2}. With the increasing number of implantees with remaining sensory cells and hearing, there is also growing interest in drug treatment for preservation and protection of remaining hair cells and their synaptic connections with the auditory nerve³. There are many potential strategies for providing local drug delivery to the cochlea (Table 1) including hydrogels, microfluidics, osmotic pumps, gene therapy/viral vectors, and micro/nanoparticles.

Although multiple local delivery strategies are under development, systemic drug delivery, either orally or intravenously⁴, has historically been the primary mode of pharmaceutical delivery to the cochlea because it is relatively safe and non-invasive. However, because of the existence of the blood-labyrinth barrier, one of the principal limitations of systemic delivery is that therapeutic levels of the drug may never reach the cochlea. Further, systemic delivery is more likely to lead to unwanted side effects, as there is increased opportunity for the drug to act on other organ systems.

Local delivery directly into the cochlea is therefore becoming a preferred strategy for the delivery of pharmaceuticals to the inner ear^{3,5}. One option is intratympanic or transtympanic delivery that involves the placement of pharmaceuticals into the middle ear and relies on diffusion across the round window membrane (RWM) for agents to reach the inner ear. Such delivery is relatively non-invasive and exploits the permeability of the RWM. A drug can be injected through the tympanic membrane directly into the middle ear or more commonly, it is placed in a release medium (i.e. biodegradable hydrogel) on or near the RWM⁶. A disadvantage for use of intratympanic delivery is that it can lead to uncertainty as to the amount of drug that reaches the cochlea due to drug losses through the Eustachian tube in the middle ear, the size and charge of delivered particles, and variations in the thickness and composition of the RWM itself⁴.

Another local option is intracochlear delivery directly into the cochlear fluids via surgical intervention. Unfortunately, the half-life of drugs directly injected into the cochlea is relatively short (minutes or hours) which limits the utility of this approach. Further, when a drug is injected, it is likely to accumulate at the site of injection rather than being distributed along the cochlear spiral. An additional intracochlear delivery method utilizes microfluidics, including osmotic pumps, reciprocating microfluidic systems, and incorporation of microfluidic channels within the cochlear implant⁴. Disadvantages of this method are that the pumps/channels must be refilled and their fill ports are susceptible to biofilm formation.

A more recent strategy for intracochlear delivery is the use of viral vectors, or gene therapy to deliver molecular therapeutics to the cochlea wherein most recent studies have focused on the use of adenovirus (Ad) and adeno-associated viruses (AAV) as cochlear delivery vehicles⁷. Though promising, this approach is not without its concerns. Specifically, the use of a virus poses significant toxicity concerns related to immunogenicity and some of the

vectors can be difficult to generate². Further, because of lack of cell targeting vectors are randomly dispersed within the cochlea; thereby limiting the number of genes that can be efficiently delivered to auditory neurons. Effective delivery to the intended target is critical in order to have the desired therapeutic impact.

Intracochlear drug delivery via a micro- or nanocarrier overcomes many of the limitations encountered with the aforementioned drug delivery techniques. In particular when placed in the cochlea, a micro-/nano-carrier enables a sustained release of drug over time (days or weeks) and could provide greater distribution and accumulation of the drug along the cochlear spiral therefore providing enhanced therapeutic benefit. As a result, polymeric micro- and nanoparticles have gained prominence as drug delivery vehicles. Several particle types have been utilized to facilitate therapeutic delivery to the inner ear including hydroxyapatite, silica, and polymeric nanoparticles⁸. Because of their wide-ranging and tunable properties, polymeric micro/nanoparticles are garnering increased interest for cochlear drug delivery. Specifically, polymeric materials such as poly-L-lysine (PLL), polylactic-co-glycolic acid (PLGA), and poly(L- caprolactone) (PCL) have been investigated as potential drug carriers to attenuate pathological conditions of the inner ear^{6,9}.

The characteristics of PLGA particles are particularly promising as they can be designed to meet many of the desired criteria for a drug delivery vehicle. These criteria include biocompatibility (PLGA is an FDA approved material and its degradation products are naturally eliminated from the body), controlled drug release (polymer degradation rates of weeks to months can be tuned to meet therapeutic needs by changing the ratio of lactic to glycolic acid), tailored size and shape enhance persistence and distribution of particles in the cochlea (resulting from control of fabrication process parameters), and potential for targeted delivery (via particle surface functionalization using the free chemical groups on the polymer surface). Moreover, polymer particles fabricated with electrohydrodynamic co-jetting (EHD) can be compartmentalized allowing loading of multiple pharmaceuticals into a single particle. Loading of multiple therapeutic agents is important to facilitate the delivery of drugs capable of attenuating the acute impact (swelling, etc.) of trauma and providing the sustained release of agents needed to potentially protect remaining hearing. Further, discrete compartments also enable pharmaceuticals with distinct pharmacokinetic profiles to be delivered from the same platform. This flexibility expands the combination of agents that can be simultaneously screened to identify the best drug combinations to ameliorate hearing loss following cochlear trauma; thereby ultimately improving the utility of the intervention. Our study tested intracochlear release of the dopamine agonist Piribedil from segmented microparticles. This glutamate inhibitor/anti-excitotoxic agent was chosen for three reasons: previous efficacy in protection from noise, current clinical use and potential for re-purposing, and inherent fluorescence¹⁰.

The use of EHD also allows for fine control of particle shape and size, characteristics that have previously been demonstrated to impact drug delivery, macrophage uptake, and cell uptake of particles¹¹. Cell binding is size and shape limited because test articles must be sufficiently small compared to a target cell. The size of target cells in the inner ear range from 8–20 μm in length, therefore a candidate microparticle with a diameter of approximately 8 μm was selected to encourage cell binding and limit particle phagocytosis

by macrophages. Use of material that forms acidic, albeit natural byproducts, in the pH sensitive fluid environment of the cochlea, was controlled by modulation of particle characteristics such as size and porosity¹². By selecting a particle size of approximately 8 μm for assessment of cochlear drug delivery, we are well below the size range of concern with respect to acid accumulation within particles¹³. Further, use of acetal dextran enables the fabrication of a porous particle, thereby inhibiting the development of an acidic particle interior because of increased exchange between the particle and the incubation medium in which it is contained. As porosity is increased, the ability to form extremely acidic microenvironments decreases because degradation products escape more readily from the interior and surrounding medium. In this study, we demonstrate the ability of dual carrier polymeric microparticles to persist and release their contents in a biocompatible manner within the cochlea.

2. Materials & Methods

2.1 Particle Materials

Dextran, chloroform, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), pyridinium p-toluenesulfonate, 2-methoxypropane triethylamine, acetic acid, sodium acetate, phosphate buffered saline (PBS), poly[(m-phenylenevinylene)-alt-(2,5-dihexyloxy-p-phenylenevinylene)] (MEHPV) as the blue marker for confocal imaging, and tween 20 were used as purchased from Sigma Aldrich, USA. Polylactide-co-glycolide (PLGA) with a molecular weight of 44 kDa and a ratio of 50:50 lactide to glycolide was purchased from Lactel Corporation. Piribedil was purchased from Ontario Chemicals.

2.2 Particle Fabrication

Particles were made from polylactide-co-glycolide and dextran acetal (PLGA/dex). Dextran acetal was chemically modified from dextran according to previously published work by Bachnelder, et al¹⁴. A light-emitting polymer, poly[(m-phenylenevinylene)-alt-(2,5-dihexyloxy-p-phenylenevinylene) (MEHPV), was also incorporated into the PLGA and PLGA/dex compartments to facilitate particle visualization via confocal microscopy. PLGA (MW: 44 kDa) with a lactic to glycolic acid ratio of 50:50 was used and PLGA/dex compartments contained 1:3 PLGA and 2:3 Dextran Acetal. All segmented particles were created via electrohydrodynamic co-jetting¹⁵, a process that involves a side-by-side capillary needle system containing polymer solutions and the application of an electric field to the system. The interface between the solutions is stabilized by the field enabling the formation of an electrified polymer jet of particles with multiple distinct compartments. Additional details on processing of the particles used in this study for cochlear delivery can be found in Supplemental information and Rahmani, et al^{16,17}. The jetted microparticles were imaged via confocal laser scanning microscopy (CLSM) using an Olympus confocal microscope at the Microscopy and Imaging Laboratory facilities at the University of Michigan. Prior to an experiment, a known mass of particles was suspended in artificial perilymph (AP; 118 mM NaCl, 30 mM KCl, 2.0 mM MgSO₄, 1.2 mM CaCl₂, 5.0 mM HEPES; pH = 7.35–7.40, osmolality = 285–294 mOsm) or artificial perilymph with guinea pig serum albumin (GPSA) to create a 15 mg/mL infusion solution.

2.3 In vivo infusion

Surgeries were conducted in a sterile environment and utilized aseptic technique. For *in vivo* infusions, Hartley guinea pigs (Charles River Laboratory, Wilmington, MA) were anesthetized and a post auricular approach was used to provide access to the middle ear. The temporal bone was drilled to visualize the cochlea and a fine pick was used to create a small hole in the basal turn of the cochlea near the round window. A microcannula with a silastic ball was inserted 0.5 mm into the basal turn of the scala tympani and cyanoacrylate was used to seal the cannula in place as outlined previously¹⁹. The microcannula was made from polyethylene 10 tubing and polyimide (I.D. = 0.12 mm, O.D. = 0.16 mm). The silastic ball was made from Sylgard. A syringe infusion pump was used to deliver either a particle solution or a vehicle solution consisting of artificial perilymph and guinea pig serum albumin into the scala tympani of the guinea pigs at a flow rate of 1 μ l/minute over 5 minutes. Infusions were always performed on the left ear and the right ear was used as needed for a contralateral control. NIH guidelines for the care and use of laboratory animals have been observed.

2.4 Harvesting, cryoprotection, and decalcification of cochlear specimens

Guinea pigs were anesthetized and euthanized by injection of sodium pentobarbital. In all cases, secondary euthanasia was performed by transecting the aorta and ventricle. Animals were then decapitated and the temporal bones that encase the cochleae were detached. Excess bullar bone was removed to facilitate visualization of each cochlea and then the middle ear bones were also detached. Specimens were fixed in 4% paraformaldehyde (PFA) for 1–2 hours. Following fixation, cochleae were decalcified in a solution that was two-thirds formic acid and one-third 7% sucrose overnight. Prior to freezing, specimens were placed in foil molds and immersed in a 30% sucrose solution. Freezing was performed by placing the bottom of the container in contact with liquid nitrogen cooled 2-methyl-butane. Specimens were wrapped in parafilm and stored at -80°C until sectioning.

2.5 Cryostat Sectioning

Samples were cut into 14 μ m sections. For stereological samples, the cochleae were sectioned up to a depth of approximately 4000 μ m. Every 6th section was collected. A random number generator was used to select a number between 1 and 6. The number generated identified the first slide for analysis in each cochlea. Thereafter, every 6th slide was evaluated such that the slides with numerical markings of 1, 7, 13, etc. were exhaustively assessed. A total of 61 slides were generated for each animal and 10 slides from each animal were assessed to ascertain particle number and distribution. For immunohistochemistry, up to 4 midmodiolar sections were taken from the MP infused cochlea of each animal. These sections were stained with CD45, a leukocyte antigen, to denote immune cell activity, and propidium iodide (PI) to indicate the presence of general cell structures such as nuclei. Cryosections of guinea pig liver were also made for use as positive and negative (in the absence of primary antibody) controls. The number of respective CD45+ and PI+ cells in cochlear cross sections were counted and the ratio CD45+ cells to total cells (CD45+ and PI+) was calculated to determine the percentage of CD45+ present within treated and untreated cochleae.

2.6 Infused particle number and persistence

A sample of the particle solution was counted before the infusions using a hemacytometer. Persistence and distribution assessments were conducted using cryosections from cochleae that had particle infusions 7 days prior to harvest of the cochlea (n=3). Sections from various depths of the cochlea were sampled for particle number. The guinea pig cochlea consists of four turns with two perilymphatic compartments, the scala tympani and scala vestibule. During assessment, the location and distribution of particles within intact cochlea and cochlear cross-sections was determined.

2.7 Stereological Analysis

Stereological analysis was used to determine particle distribution in animals infused with unloaded PLGA/dex particles (n=3) and to estimate the number of particles entering the cochlea at the time of infusion. In particular, the optical dissector method was used to systematically create image slices that contained particles in tissue at multiple planes within the cochlea. This technique is an unbiased method whereby an object is counted the first time it appears in an image¹⁸. A confocal microscope with a 405 nm laser was used to acquire z-stack image series of particles within cochlear cross-sections. The z-step size was 3.6 μm . Every 6th slide from each sample was evaluated using an unbiased counting grid composed of green inclusion lines and red exclusion lines. The grid was superimposed on top of specimen images and a particle was counted if it was inside of one of the squares in the counting grid or in contact with a green inclusion line. The total particle number within an infused cochlear sample could be estimated by multiplying the number of particles counted within the assessed samples by a factor of 6.

2.8 Auditory Brainstem Response (ABR)

Animals were anesthetized with xylazine (10 mg/kg intramuscularly) and ketamine (40 mg/kg intramuscularly). Needle electrodes (active, reference, and ground) were inserted subcutaneously at the vertex and below each pinna and used to record the neurologic response. Up to 1024 responses were averaged for each stimulus level, with the stimulus consisting of a 15-msec tone burst, provided at 10/sec. Pure tones were delivered via a transducer coupled to the external auditory canal at 4, 8, and 20 kHz. Initial sound levels were set at 80 dB for pre- and post- infusion tests. Threshold determination was made by non-blinded evaluators based on the visual detection of maximum peak–peak amplitude of the resulting waveforms. ABRs were performed prior to infusion to enable exclusion of animals with abnormal hearing, and to enable detection, if present, of threshold shifts post-infusion.

2.9 Hair cell counts

To prepare specimens for hair cell analysis, ears were harvested in the same manner as those used for cryosection preparation with a few notable differences. Following removal of the middle ear bones, the apex was visualized under stereoscopic magnification and slightly perforated with a 28G needle to create a small hole. Then the round window was opened and approximately 300 μL of 4% PFA was infused directly into the cochlea via the hole in the apex. Specimens were postfixed by immersion in 4% PFA overnight. The following day,

cochleae were rinsed and the otic capsule, lateral wall, and tectorial membrane were carefully removed. Phalloidin was used to stain the modiolous and the attached organ of Corti. Following rinsing to remove excess stain, the organ of Corti was dissected from the modiolous and each turn was mounted onto a microscope slide and coverslipped. Phalloidin staining of the organ of Corti enabled visualization and counting of both inner and outer hair cells (as indicated by the presence of nuclei and/or stereocilia). The blinded counts were performed as described previously²¹. Counts were then plotted using a cytochleogram program developed in house²⁰, and depicted as percentage hair cell loss at a particular distance from the apex as compared to a database of normal guinea pigs (those not exposed to any external stimuli or agents that could induce hearing loss). Tracking distance along the cochlear spiral also facilitated the correlation of areas of loss with known frequency maps of the guinea pig cochlea. This provided insight on areas that may be functionally affected by the treatment.

2.10 Immunohistochemistry

Specimens were prepared similarly to those used for cytochleograms, except post fixation was performed for 2 hours rather than 12. Then the same protocol was followed as outlined in 2.4. Midmodiolar sections, 12 in total, were selected from 3 of the animals used for the collection of Piribedil exposed perilymph *in vivo*. These sections were assessed with CD45, a leukocyte antigen, to determine the extent to which particle infusion induced a local immune response. Sections from contralateral control ears and the liver from one of the guinea pigs (in the absence of primary antibody incubation) served as negative controls. In the presence of CD45 primary antibody, cryosections of the liver were used as positive controls. The specimens were pre-treated for 15 minutes with 0.3% Triton X-100 in PBS, followed by PBS rinsing (3×5 minutes). Each section was blocked for 1 hour with 5% goat serum, permeabilized for 30 minutes with 0.3% Triton in 5% goat serum, and incubated with a primary antibody for CD45 (mouse anti-guinea pig), and PBS (1:50). The diluted primary solution was incubated with plates overnight at 4°C in a sealed chamber.

2.11 Statistical analysis

An analysis of variance (ANOVA) was performed on the hair cell counts of MP infused cochleae to determine whether significant quantitative differences existed between sensory cell viability in treated and untreated ears. Using SPSS software, a two factor model, with factor 1 = ear and factor 2 = turn was employed to assess whether any observed differences in hair cell loss were significantly different between treated and untreated ears and between turns. Further bonferroni post-hoc correction was used to account for the multiple comparisons made. The p value for significance was .05.

2.12 In vivo Piribedil release

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed to determine the Piribedil concentration in cochlear fluid samples. Use of LC allows separation of the drug from a biologically relevant background matrix (native or artificial perilymph) and mass spectrometry enables detection at very low concentrations. The extraction solvent contained 997.5 µL acetonitrile + 2.5 µL Acar mix. Six standards (0–3000 nM), were prepared by spiking native or artificial perilymph with 3 µM stock Piribedil solutions that

had been diluted with the extraction solvent. Experimental samples obtained from animals exposed to Piribedil-loaded microparticles were mixed with extraction solvent and vortexed, followed by 5-minute incubation at 4°C. Two vortex/cooling cycles were completed prior to centrifugation of the samples at 15,000 rpm and 4°C for 5 minutes. Sample supernatants were transferred to autosampler vials and subjected to LC-MS analysis utilizing an Agilent 1200 RRLLC coupled to an Agilent 6410 Triple Quad LC/MS. Analyte peaks were resolved with the use of a Waters Xbridge C18 (50 mm × 2.1 mm, particle size 2.5 µm) column. The liquid chromatographic conditions were as follows: mobile phases: A = 5mM ammonium acetate, adjusted to pH 9.9 with ammonium hydroxide; B = acetonitrile; flow rate: 0.25 mL/min; and injection volume: 2 µL. The gradient began at 25%B and followed a linear regime from 25% to 75%B over 5 minutes before increasing to and holding at 100% for 3 minutes. Then it was returned to 50%B and re-equilibrated for 4 minutes, thereby resulting in a total run time of 12 minutes/injection. The mass spectrometry source conditions were: gas temp: 325°C, gas flow: 10 L/minute, nebulizing pressure: 40 psi, and capillary voltage: 4000 V. All mass spectrometry readings were collected in positive ion mode. Though the total volume of perilymph in the cochlea is 10 µL, the volume of perilymph in the scala tympani, the chamber into which the particle solution is infused, is only 4.7 µL⁵. The remaining fluid consists of fluid from the scala vestibule or cerebrospinal fluid therefore a simple correction factor is attained by dividing total perilymph volume by collected perilymph volume.

3. Results

3.1 Particle Fabrication

The PLGA/dex particles were fabricated as described previously in Rahmani, et al^{16,17}. Average particle diameter was 7.7 ± 0.12 µm and was determined from ImageJ analysis of scanning electron microscope (SEM) images of the microparticles. Further characterization of the segmented microparticles, such as zeta potential measurements, morphology, and size distribution can be found in Supplemental information and Rahmani, et al^{16, 17}.

3.2 Infused particle number and persistence

The number of particles released into the cochlea at the time of infusion was examined. On average, 350,000 particles were infused into each cochlea as counted by a hemacytometer. The persistence of the aforementioned infused particles within the cochlea was also examined. On day 7 following microparticle infusions, untargeted unloaded particles were distributed in the cochlea (Figure 1). The vast majority of particles were located in the first turn of the cochlea ($94 \pm 17\%$), followed by the second turn ($6 \pm 2\%$) and the third and fourth turns (0%). The third and fourth turns were combined during analysis due to the relatively few numbers of particles found in each turn alone.

3.3 Stereological Analysis

In order to facilitate an estimation of the remaining particle number, it was necessary to use stereology, an analysis method that utilizes random, systematic sampling to count objects, in this case, fluorescent particles within cochlear cross-sections. Though methodological restraints prevented retroactive analysis of the samples discussed in section 3.2, particle persistence and distribution of one timepoint, 7 days, was used for stereological analysis. In

the new group of animals (n=3), the distribution profile was similar to the previous samples and the average number of particles persisting after 7 days was 20,000±4000. No particles were found in the contralateral ear and a comparison of the number of persisting particles to the number of infused particles demonstrated that approximately 5.7% of untargeted particles were retained in the cochlea following infusion.

3.4 Auditory brainstem responses (ABRs)

The hearing thresholds of the animals used in this study were assessed both pre- and post-infusion. ABRs were administered before the start of the experiment to promote the inclusion of only those animals with behaviorally normal hearing (particularly in the lower two-thirds of the cochlea; the upper third was beyond the frequency threshold that can be measured non-invasively). Baseline hearing assessment also provided a basis for comparison to ABR results following infusion to assess particle impact on hearing. Post-infusion testing was conducted at the same three frequencies, 4, 8, and 20 kHz as the pre-test. If the intensity level required for the subject to detect the stimulatory tone at any of the frequencies changed, then a threshold shift occurred. Hearing was considered to have worsened if the sound intensity needed for the subject to detect the stimulatory tone was more than 10 decibels higher than in the pre-test. This value was selected because it has been shown to be outside the bounds of normal experimental variation and therefore can be indicative of an actual functional change in hearing capacity²². A total of eight animals underwent ABR assessment, five that received microparticle infusions and three that were infused with a vehicle solution consisting of artificial perilymph and guinea pig serum albumin. As seen in Figure 2, following microparticle infusion, three out of five animals had hearing within the normal range as compared to their pre-infusion values that is indicated by the threshold shift. One of the MP infused animals, GP 1, demonstrated a threshold shift (>10 dB) at one frequency, 20 kHz. Another MP infused animal, GP 3, demonstrated threshold shifts at all frequencies. Following vehicle infusion, only one animal, GPV 2, demonstrated a large threshold shift at 4 kHz (Figure 2). Therefore, it was imperative to evaluate these results in conjunction with hair cell viability, a morphological indicator of cochlear health, to enable the most complete interpretation of experimental outcomes.

3.5 Cytocochleograms

In addition to functional performance, cochlear cell/tissue appearance, particularly the presence or absence of hair cells and immune cells, was evaluated following microparticle and vehicle infusion. Cytocochleograms provided a visual representation of areas of missing hair cells and the location of these cells relative to the apex and base of the cochlea. All infused cochleae were assessed via cytocochleogram and contralateral cochleae were evaluated as needed. Based on previous studies, an area was considered normal if the outer hair cell (OHC) loss occurred intermittently and was 20% or less²³. Further, there should be almost negligible loss of inner hair cells (IHCs) observed. It should be noted that in the Hartley strain of guinea pig used in these studies, it is not uncommon to have untreated animals that have large amounts of hair cell loss in the apical third of the cochlea that cannot be detected by ABR during prescreening [unpublished observation] because of the technical limitations of the testing equipment. The distance from the aforementioned area is sufficiently far however, from the lower sixth of the cochlea where the particles were infused

to enable the determination of the impact of particle delivery on hair cells/hair cell viability. Upon assessment, two of the microparticle infused animals used for cytochromeograms (GP 1 and GP 4) were found to have the aforementioned apical hair cell loss. However, in all but one case, microparticle infused animals had hair cell losses in all rows of less than 20% in the areas adjacent to the site of infusion (Figure 3). Further, the cytochromeogram of GP 3 demonstrated minimal hair cell loss; therefore, functional deficits identified at the lower frequencies during ABR were likely the result of conductive hearing losses due to the presence of middle ear fluid that was detected during gross dissection. Therefore, microparticle infusion was well tolerated in four out of five animals with only one animal, GP 1, displaying functional and histological losses. For vehicle infused animals, two of three animals, GPV 1 and GPV 3, did not exceed the functional loss criteria as determined by ABR (Figure 2). Further, for GPV 2, the decrement seen at 20 kHz could be explained by the presence of scar tissue, particularly when evaluated in conjunction with the hair cell counts obtained for that animal. Upon histological evaluation by cytochromeogram, all of the vehicle infused animals had minimal to limited hair cell losses near the cochleostomy, including GPV 2, which had hair cell losses in all rows of less than 20% in the areas adjacent to the site of infusion (Figure 4). A moderate apical hair cell loss was also demonstrated in GPV 3, one of the vehicle infused animals (Figure 4). In addition, within MP infused animals, a 2-factor analysis of variance (ANOVA) on OHC loss determined that both ear and turn losses were significant with a p-value below the $\alpha=.05$ level indicating that treated ears and locations in the higher turns demonstrated greater losses. Subsequent analyses compared the OHC loss of treated and untreated ears at each individual turn (Figure 5). OHC loss in turn 1, near the infusion site, was not significantly different between treated and untreated ears. In turn 2, the difference was statistically significant at .02, however, as average losses for both the non-treated and treated ears were well under 10%, the difference may not be as functionally meaningful. Interestingly, turns 3 and 4 were significantly different when treated (left ears) are compared to non-treated (right ears). It is likely however, that differences observed are related to factors other than the physical presence of the particles because distribution studies demonstrated that particles were primarily located in the first and second turns.

3.6 Immunohistochemistry

White blood cells were present in both treated and non-treated ears. The cells were primarily located near vasculature and within the spiral ligament. This finding correlates well with other work reporting the presence of macrophages within the native cochlea²⁴. Further, the number of white blood cells present in treated ears ($7.21\% \pm 4.62$) was comparable to that seen in the untreated ears ($6.57\% \pm 3.38$) indicating that at 7 days post-infusion, the immune response to the particles was negligible (Figure 6).

3.7 In vivo Piribedil release

Piribedil-loaded microparticles contained 2 w/w% Piribedil and a 7-day post-infusion timepoint was selected to perform perilymph sampling to ascertain *in vivo* concentration of Piribedil release from the particles. The total volume of fluid collected ranged from approximately 6.9–10.2 μL and Piribedil was detected in all analyzed samples (Table 2).

Although Piribedil was present in all samples, the apparent concentrations of the drug at 7 days post-infusion were below therapeutic level¹⁰.

4. Discussion

Historically, much of the focus with regard to cochlear drug delivery has been via systemic administration or acute injection of free drug. Carriers, particularly microparticles made from biodegradable, tunable polymers have the potential to facilitate sustained local drug release to the cochlea. In this study, the polymeric microparticles used were observed to accumulate primarily within the first turn. This finding was not unexpected as this location was the site of infusion and existence of fluid flow in the cochlea is negligible. During infusion, the dispersion of particles to other turns relies primarily upon diffusion and the small flow induced by the micropump. The infused particles were found in comparable numbers and distributions 1 and 7 days post-infusion thereby indicating that particles capable of surviving acute clearance (washout via eustachian tube, immune response, etc.) were able to remain in the cochlea for an extended period. Further, no particles were detected in the contralateral ear, indicating that the delivery platform does not diffuse to the contralateral cochlea via cerebrospinal fluid, nor were they seen in the liver, as can occur with nanoparticles²⁵, thereby eliminating concerns about inadvertent effects. Though the percentage of remaining particles was small ($5.7\% \pm 1.1$), the utility of delivery from particles following infusion would be dependent on the dose of drug needed to achieve therapeutic level and the extent to which the drug was able to be incorporated (weight %) into the particles. Further, the percentage observed could be a consequence of the sample processing required to generate the particle cross-sections used in the analysis. During incubation in the decalcifying solution and/or the cryoprotection solution, particles located in the fluid spaces could be displaced, meaning that only particles closely associated with the lining of the cochlear chambers would remain for analysis. Therefore, it is possible that non-adherent particles were simply “washed out” of the system before they could be assessed. Potential modifications to the surface of the microparticles to include ligands targeting cochlear structures or implant materials may also help to increase the number of attached particles.

The majority of infused animals had comparable or only slightly elevated post-infusion hearing thresholds and minimal hair cell loss following infusion. Hair cells act as mechanotransducers and enable the conversion of fluid movement into electrical signals within the cochlea that can then be interpreted by the brain. These cells are sensitive to damage from both chemical and physical means and their absence impairs auditory function. One of the microparticle infused animals with an apparent post-infusion threshold shift was GP 3. This animal had an observable threshold increase at all frequencies, however the greatest shifts occurred at 4 and 8 kHz; this physiology correlated well with its gross anatomy in that fluid was present in its middle ear and excess fluid makes it more difficult for sound to be transmitted, particularly at lower frequencies. Further, GP 3 had similar numbers of viable hair cells as non-shifted animals, therefore conductive losses rather than particle presence caused the detected functional deficits. The almost negligible loss of inner hair cells and normal losses of outer hair cells near the site of infusion indicate that locally delivery of segmented microparticles was well tolerated in four out of five microparticle

infused animals. One animal, GP 1, however, did experience functional and histological detriment following MP infusion. Within the three vehicle animals that received an infusion of the vehicle solution consisting of artificial perilymph and guinea pig serum albumin, one had an apparent threshold shift. This animal, GPV 2, had a large (greater than 40 dB) threshold shift at 4kHz. Examination and analysis following harvesting of the cochlea GPV 2 revealed a normal cytochleogram and the presence of scar tissue on the treated cochlea although the tympanic membrane and middle ear were clear. This finding indicates that the functional deficit observed is also likely the result of conductive losses. The two remaining vehicle infused animals tolerated the procedure well and did not have extensive amounts of hair cell loss near the site of infusion.

Upon further examination of the ANOVA analysis, the determination that both ear and turn were significant factors in predicting hair cell loss was found to be primarily based on differences between OHC presence in turns 3 and 4 of treated ears. The statistically significant difference observed in treated versus untreated ears in turns 3 and 4 seems unusual because distribution studies determined that the majority of particles were in the first and second turns. Further, based on the site of infusion, any potential hair cell loss would be expected to occur at the base of the cochlea. It is possible that the differences seen are related to an increase in intracochlear pressure induced by surgery or the viscosity of the delivery solution formed from artificial perilymph and guinea pig serum albumin as this increased trauma was also present when a vehicle solution was used. To alleviate potential pressure changes caused by displacement of native perilymph during infusion, an outlet hole could be drilled to provide an alternative exit to the cochlear aqueduct, thereby enabling removal of excess fluid from the perilymphatic space²⁶. Further, future work could include assessment of the functional and pathophysiological impact of the viscosity of the delivery solution and incorporate this knowledge into future iterations of the particle delivery protocol. In addition, surface modification could enable particle attachment to implants and eliminate need for a delivery solution.

The apparent biocompatibility of PLGA/dextran microparticles with the cochlea was not unexpected due to the composition of the constituent materials. PLGA is a FDA approved biodegradable polymer and it, along with its degradation products, is relatively biologically inert¹⁴. In addition, Dextran is a natural polysaccharide that is also well tolerated in the body¹⁴. Further, the detection of Piribedil, a proof of principle medication in this study, demonstrated that drug release from the particles is measurable albeit currently sub-therapeutic within cochlear fluids at 7 days post-infusion; Piribedil has poor solubility that resulted in low percentage incorporation into the polymer matrix of the microparticle. An alternative non-competitive glutamate agonist with better solubility could be utilized in future work to address the efficacy of intervention, thereby increasing the likelihood that therapeutic level could be attained *in vivo*.

5. Conclusions

This work has demonstrated the feasibility of delivering microparticles with multiple distinct compartments to the cochlea *in vivo*. Specifically it has characterized the *in vivo* persistence, distribution, and drug release from the chosen particle system. Particles persisted within the

cochlea for at least seven days. Following particle infusion, there was only a slight impact on cochlear functionality in the majority of animals and a robust immune response was not induced. Cell survival and morphology were also well maintained in areas of the cochlea with high numbers of particles. This technology represents a tunable platform with potential for intracochlear drug delivery as targeted free particle suspensions or particles attached to an auditory implant. Future work should consider the impact of the viscosity of the particle delivery solution on cochlear functionality and assess efficacy of drug delivery from segmented particles in a trauma model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Particle distribution of unloaded microparticles observed 7 days after *in vivo* infusion. A) Percentage distribution of particles within the first four turns of the guinea pig cochlea where turn 1 (B–A) is the closest to the cochleostomy/infusion site and turn 2 (B–B) is the next adjacent turn.

Figure 2.

Cochlear function 7 days post MP or vehicle infusion as represented by auditory brainstem responses. For all animals, threshold shift (difference between pre- and post-infusion thresholds) at 4, 8, and 20 kilohertz are shown. A threshold shift greater than 10 dB (*) is indicative of a change in an animal's hearing. At the majority of frequencies across animals, no shift is observed, though at 20 kHz GP 1 and 3 both demonstrate shifts and GP 3 also demonstrates shifts at 4 and 8 kHz. Among vehicle infused animals, GVP 2 demonstrates a notable shift at 4 kHz. Based on anatomical observations, the threshold shifts observed at lower frequencies are likely the result of conductive losses rather than the physical presence of the microparticles. Threshold shifts for GP and GVP animals are denoted by patterned or solid columns, respectively. GP= microparticle infused animals. GVP= vehicle infused animals.

Figure 3.

Cochlear hair cell death as represented by cytochleograms (graphical representation of hair cell loss) at day 7 post-infusion for microparticle infused guinea pigs (n=5) receiving 5 μ L of microparticles. The regions of auditory brainstem response (ABR) functional testing as well as the cochleostomy site have been indicated along the x-axis. In all animals, hair cell loss near the cochleostomy site in the infused ear is minimal. Cytochleograms for all left/treated ears are shown and a representative cytochleogram (GP 1) of a right/untreated ear is provided in the upper right panel of the figure.

Figure 4.

Cochlear hair cell death as represented by cytocochleograms (graphical representation of hair cell loss) at day 7 post-infusion for vehicle infused guinea pigs (n=3) receiving 5 μ L of vehicle solution. The regions of auditory brainstem response (ABR) functional testing as well as the cochleostomy site have been indicated along the x-axis. In all animals, hair cell loss near the cochleostomy site in the infused ear is limited. Cytocochleograms for all left/ treated and right/untreated ears are shown.

Figure 5.

Cochlear outer hair cell loss comparison. Graphical depiction of the OHC loss as a function of ear (treated/left vs. non-treated/right) and turn at 7 days post microparticle infusion. A two-factor analysis of variance (ANOVA) with $\alpha=.05$ found that turn ($p=0.00$) and ear ($p=0.00$) were both significant. The differences between ears were primarily driven by OHC losses in turns 3 and 4. Differences between ears in turn 2 were functionally negligible and did not exist in turn 1. OHC= outer hair cell. Black bars are standard deviation.

Figure 6.

CD45 presence in treated and untreated cochleae. Resident white blood cells may be in the normal cochlea and no differences are seen in the number of these cells present at 7 days following MP infusion. A) A representative cochlear cross-section wherein leukocytes are identified as nuclei (red) whose surface/cell membrane is positive for CD45 (green). B) Quantification of the percentage of CD45+ cells present within treated and untreated cochlear cross-sections. Black bars are standard deviation.

Table 1
Potential Strategies for Local Cochlear Drug Delivery

Contains a synopsis of various platforms investigated for local cochlear drug delivery and outlines the advantages, limitations, and distribution profile of each type of carrier/strategy.

Potential Inner Ear Carriers	Advantages	Limitations	Cochlear Distribution (turn)
Hydrogel/Sponge	Ease of insertion/non-invasive; biodegradable	Extracochlear/Intratympanic application; drug incorporation by diffusion; relies on RWM permeability	Basal
Microfluidics/Mini-Osmotic Pumps	Continuous delivery; real time modulation of infusion rate	Must be refilled/size; biofilm formation; finite power supply	Basal to apex
Viral Vector	Induce regeneration	Induce immune response; toxicity	Variable
Biodegradable Micro/nanoparticles	Biodegradable; size; multifunctional	Invasive; release rate predetermined	Variable

Table 2
Intracochlear Concentration of Piribedil at 7 Days Post-infusion of Piribedil-loaded Microparticles

Contains the volumes, apparent concentrations, and volume corrected concentrations from each sample. The corrected concentrations were obtained by multiplying the apparent concentration by a correction factor derived from dividing total perilymph volume by collected perilymph volume.

Specimen	Volume (μL)	Apparent Concentration (nM)	Volume Corrected Concentration (nM)
GP 11	10.20	339.23	736.20
GP 13	9.00	106.04	207.57
GP 22	8.40	207.41	370.68
GP 25	7.22	205.73	316.04
GP 15	6.88	223.43	327.07
GP 26	7.10	192.01	290.06
Average	8.13	212.31	374.60
Standard Deviation	1.31	74.84	185.20