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Dual Release Carriers for Cochlear Delivery

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In memory of Prof. KahpYang Suh who was a great scientist and friend

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Deafness is the number one disability in the US,^[1] with over 17% of the population, and half of those over the age of 75, diagnosed with some level of hearing loss.^[2] Given the large number of patients, and their foreseeable rise as the elderly population grows, it is imperative to develop medical technologies that can restore hearing.

Cochlear implants are used to directly stimulate the auditory nerves (spiral ganglion neurons, SGN) and return some level of hearing in patients where hair cells are either severely damaged or completely destroyed.^[1,3] Due to the success of cochlear implants, these devices have increasingly been used in patients with a lower degree of hearing loss, where a subset of the total hair cell population is still functional.^[1,4] Unfortunately, the trauma associated with cochlear implants can increase the level of ROS (reactive oxygen species) and may cause damage to the remaining hair cells.^[4,5] Hair cells produce multiple neurotrophins and their loss results in the reduction of these molecules in the ear.^[6] A reduction of neurotrophins within the cochlea can then lead to the degeneration of the SGN, subsequently compromising the functionality and effectiveness of the cochlear implant.^[7]

Recent studies have thus focused on the use of ROS-preventative therapeutics to reduce the trauma from implant insertion.^[8] While traditional therapies have relied on systemic administration of therapeutics, most recent strategies have focused on local delivery to better achieve therapeutic levels in the cochlea without causing side effects that are prevalent in systemic administration.^[9] Therapeutics used for the prevention of hearing loss include antioxidant and antiexcitotoxic drugs,^[5,10–12] heat shock proteins,^[13] neurotrophic factors,^[14–17] and gene therapy.^[18] However, the use of heat shock proteins and gene therapy has proven to be difficult due to the incomplete understanding of the signaling pathways involved or the inability to target the therapies to specific sites in the cochlea,^[19] while the use of antioxidative/antiexcitotoxic drugs and neurotrophins has shown initial success.^[10,14] Specifically, it has been shown that a short-term application of antiexcitotoxic drugs can eliminate the effect of insertion trauma on hair cells,^[20] while a long term application of neurotrophins can result in the protection and survival of the hair cells and leads to the preservation and elongation of SGNs.^[14,21]

A variety of methods have been developed to locally deliver therapeutics before, during, or after the implantation of the device. For instance, Kanzaki et al. has shown that the infusion of adenoviral vectors encoding a transgene for GDNF (glial cell derived neurotrophic factor) at the time of surgery could significantly improve hair cell survival over a 43 day period.^[22] Yamagata et al. used a cannula-osmotic pump system loaded with BDNF (brain derived neurotrophic factor) with similar results.^[17] Translating these methods to humans, however, has proven to be difficult: The use of osmotic pumps is cumbersome and prone to cause infections, while the adenoviral vector treatment poses unknown side-effects.^[1] The use of a delivery system capable of the continuous release of both, neurotrophic factors and antiexcitotoxic/antioxidant drugs, could prove to be a superior solution.

Herein, we propose the design of bicompartamental microparticles capable of encapsulating and releasing two distinct therapeutics to achieve independent release profiles. There are several parameters that needed to be considered in the design of a carrier system for cochlear delivery: i) The persistence of particles in the cochlea for the duration of the therapy; ii) the

incorporation of an antiexcitotoxicity or antioxidant therapeutic to reduce trauma-induced apoptosis and the release of the therapeutic over a 2–4 week period; and iii) the encapsulation, protection, and release of a neurotrophin to help with hair cell and SGN survival and elongation for a greater than one month period. Based on these design parameters, we fabricated micro-particles composed of two distinct compartments through the electrohydrodynamic (EHD) co-jetting technique.^[23,24] During this process, the polymers are dissolved in organic solvents and are flown in a laminar regime through metallic capillaries. To fabricate distinct compartments, multiple capillaries can be used, where each capillary can contain a different set of polymers. An electric field is applied to these flowing polymer solutions, which create a Taylor cone at the tip of the capillaries. The solutions then elongate at a rapid rate into a jet, forming individual droplets that are attracted toward the counter electrode. Due to the high speed of the jet and the rapid increase in the surface area to volume ratio, the solvents evaporate instantly, leaving behind solid particles on the counter electrode. Here, the particles retain the geometry of the original capillaries used because the polymers do not have sufficient time to mix in the short period of the production. To ensure persistence of the particles in the cochlea during the therapy, the polymers chosen for all compartments were biocompatible or biodegradable, and their degradation rates exceeded the time requirement anticipated in the cochlea. Additionally, the large size of the particles at 6–8 μm was chosen to delay uptake by macrophages.^[25]

To address the second and third criteria, particles with two distinct compartments were designed where each compartment could be individually programmed. As demonstrated in Scheme 1, one compartment was composed of PLGA and an antiexcitotoxic therapeutic piribedil, and could potentially release the therapeutic in a 15–20 day period based on the degradation rate of the polymer. The second compartment, which was larger in size than the first compartment, was composed of PLGA, polymer **1**, and polymer **2** and was used as the compartment to encapsulate and release the neurotrophin GDNF. The direct incorporation of GDNF in the jetting solutions would result in the denaturation of the neurotrophin in the organic solutions used and, while some studies have successfully incorporated similar proteins in PLGA particles, the degradation of the polymers and the associated acidic pockets typically denature the proteins during the release phase.^[26] To address this challenge, the particles were functionalized with heparin, which is well known for its interactions with various growth factors through the electrostatic attraction between specific sequences in the heparin polymer (based on specific monosaccharide and sulfation patterns), as well as its negative charge, and the corresponding groups in the growth factors.^[27,28] Heparin could be employed for the loading and release of GDNF as well as its ability to shield and biologically stabilize the GDNF during this time.^[29] To functionalize the particles with heparin, polymer **2**, a benzophenone-functionalized PLGA, was incorporated into the particles and was used to immobilize the heparin to the surface via photo-reactive chemistry.^[30] Additionally, a larger compartment size and surface area were employed to maximize the amount of heparin, and thereby GDNF, incorporated into the particles. Particles with two nonequal compartments were fabricated by using three laminar polymeric flows instead of two flows in the EHD co-jetting procedure, where two of the flows were identical. In this way, the resulting particles contained three compartments, where one was distinct while the other two were identical in chemical makeup and thus could be used for

the same functionalities. To increase the surface area, polymer **1**, a pH responsive protected dextran, was incorporated into the larger compartment. Following incubation in a low pH environment, polymer **1** was deprotected and the resulting water-soluble dextran polymer leached out of the particles creating a porous structure with an increased effective surface area. The porous structure could then be used to incorporate a larger quantity of heparin into the particles (Scheme 1). In order to achieve the maximum loading of the therapeutics and their distinct release profiles, we employed a bicompartamental design over a single compartment (isotropic) particle design, where both piribedil and GDNF would be in the same compartment: In a single compartment particle, the release of the dextran from the particles would have caused the simultaneous rapid release of the majority of piribedil from the particles during the fabrication process; while not using polymer **1** to increase the surface area would have reduced the amount of GDNF incorporated into the particles.

To characterize the designed particles, first unloaded particles fabricated through the EHD co-jetting process were analyzed for their bicompartamental nature, size, zeta potential measurements, and porosity (Figure 1). The particles were analyzed using confocal laser scanning microscopy (CLSM) to demonstrate their multicompartamental nature. As shown in Supplemental Figure 1, Supporting Information, the particles contained green and blue dyes in separate compartments (Supplemental Figure 1A–B, Supporting Information) that did not overlap when the images were combined (Supplemental Figure 1C, Supporting Information), demonstrating that the dyes were in fact in individual, distinct compartments. The size distribution of the microparticles (Figure 1B) was relatively uniform at 7.8 ± 1.4 μm and, similar to previously published results from our group,^[24] the as-fabricated particles had a negative zeta potential measurement ($-25.5 \text{ mV} \pm 0.8$) due to the free hydroxyl groups available in polymer **1** (Figure 1A). Upon pH 5 incubation of the particles and the subsequent release of the dextran, the zeta potential value of the particles became less negative ($-13 \text{ mV} \pm 2.8$), confirming the release of dextran from the particles. Furthermore, scanning electron microscopy (SEM) images of the particles (Figure 1C) clearly demonstrated the formation of pores over a period of time. Here, the level of porosity at 10 hours was chosen for future studies since longer incubation in pH 5 resulted in the degradation of the porous compartment. SEM images of longer incubation times of 15 and 20 hours are displayed in Supplemental Figure 2, Supporting Information.

Once the unloaded particles were fully characterized, their persistence within the cochlea was examined. This was especially important since the persistence of the particles and the continuous release of the therapeutics within the cochlea is crucial for the survival of the hair cells, the SGN, and thereby any resulting potential improvements in hearing. Particles were injected at the base of the cochlea and after 24 hours the animals were sacrificed and their cochlea were dissected and imaged via 2-photon microscopy (Figure 2). The 3D reconstruction of the 2-photon images from the basal section of the cochlea in Figure 2A-2 clearly demonstrates the presence of particles within the cochlea after 24 hours. Furthermore, the cochlea were sectioned and the particles in each individual turn (Figure 2A-1) were counted from three sections in the center of the cochlea for three individual animals. The table in Figure 2 displays the distribution of the particles within the cochlear turns, while the images in Figure 2B-1–4 are representative of the particle distribution in

each turn. Based on these results, the majority of the particles were located near the base (Turn 1) of the cochlea, followed by lower percentages in the turns closer to the apex.

After characterizing the unloaded particles and establishing their persistence in animals, we focused on the incorporation and release of the therapeutics from the bicompartamental particles. As mentioned previously, for the antiexcitotoxic therapeutic piribedil a continuous release over a 2–4 week period is optimal following the insertion of the cochlear implant to reduce the trauma to the hair cells. As such, a 44 kDa PLGA polymer with a degradation rate of 3–4 weeks (based on manufacturer's specifications) was chosen as the base polymer. Piribedil was loaded into the particles by directly incorporating it into the jetting solutions at a 2% w/w of the PLGA compartment (0.83% w/w of the particle). Particles containing piribedil and PLGA in one compartment were fabricated based on Scheme 1, where the particles contained a green dye in the piribedil containing compartment and a blue dye in the PLGA/polymer **1** compartments. Figure 3A demonstrates the compartmental nature of these particles based on CLSM analysis. Piribedil autofluoresced in the red channel, and this was used as a method to confirm the location of the drug. As can be seen in Figure 3A-3 and 4 images, the red channel overlaps with the green channel (middle, Figure 3A-3) and is completely separate from the blue channel (right, Figure 3A-4), confirming the encapsulation of the therapeutic in the selected compartment. To determine the release kinetics of piribedil, particles were incubated in physiological conditions and at predetermined intervals samples were taken of the released material and measured via UV visual spectroscopy at 286 nm. The release of piribedil from the particles was continuous over a 14 day period (Figure 3B), which is within the desired window for the release of the antiexcitotoxic therapeutic. The release mechanism of piribedil from the particles is similar to the release of low molecular weight molecules from PLGA microparticles where the release is a function of both the diffusion of the therapeutic from the polymer matrix and the degradation of the PLGA polymer.^[31]

Next, the incorporation of the neurotrophin GDNF was explored. Multicompartamental particles as designed in Scheme 1 with polymers **1** and **2** in one compartment were fabricated through the EHD co-jetting procedure. The particles were incubated in an acidic environment for 10 hours to create pores in the particles and then incubated in a heparin solution and exposed to UV light to immobilize the heparin to polymer **2** via the photoreactive side groups (Figure 4). Zeta potential measurements confirmed the attachment of the heparin to the surface of the particles: the as-fabricated particles had a negative zeta potential value, which decreased as the dextran was released from the particles (similar to Figure 1), and then became more negative after the attachment of the heparin (−34.4 mV) due to the negatively charged groups of the heparin polymer (Figure 4A). After heparin attachment, the particles were incubated with GDNF to incorporate it into the particles, washed to remove the unbound material, incubated with a primary antibody selective to GDNF (GDNF antibody, rabbit polyclonal IgG), and then labeled with a corresponding fluorescent secondary antibody (goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 647 conjugate). The particles were imaged via CLSM to confirm the incorporation of GDNF onto the particles (Figure 4B), where the PLGA compartment contained a green dye and the GDNF was labeled with a magenta dye. Figure 4B-1 is a CLSM image of the particles

showing the incorporation of GDNF on one side. The 3D reconstruction of the CLSM images clearly displayed the compartmentalization and presence of GDNF on the particles (Figure 4B-2).

To determine the release period of GDNF from the carrier system, particles were loaded with GDNF, washed to remove unbound material, and incubated at physiological conditions. The loading of GDNF into the particles for the release studies was done at $0.24 \mu\text{g mg}^{-1}$ of particles (0.024% w/w), as measured after the multiple washes. At various time points, the samples were centrifuged and the supernatant containing the released GDNF was collected and frozen. The samples were then measured via ELISA (enzyme linked immunosorbent assay) to determine the precise amount of GDNF released at each time point. Figure 4C demonstrates the prolonged release of GDNF over a 50 day period, which outlines the successful and continuous release of the neurotrophin at therapeutic levels.^[12,15] This overall release mechanism is a combination of the diffusion effect of GDNF from the heparin matrix of the particles and the dissociation/association of the GDNF with specific regions of the heparin polymer.^[32] During the initial release phase (days 0–5), the higher concentration of the GDNF in relation to the heparin results in a burst release of the growth factor from the particles due to simple diffusion mechanisms. As the ratio of GDNF to heparin decreases, the unbound (dissociated) growth factors quickly reassociate with free heparin groups and as a result, their diffusion out of the matrix is delayed resulting in the prolonged release seen from day 5 to the end of the study at day 50.^[28,33]

Multicompartmental particles loaded with multiple therapeutics could be an ideal carrier system for delivery to the cochlea where the distinct release of each factor is desired. In this paper, the design of particles loaded with a small molecule drug and a neurotrophin for cochlear delivery were explored. The characterization of the base particles and the in vivo persistence and distribution of the particles throughout the cochlea after a 24 hour period were demonstrated. Additionally, the selective loading of a small molecule therapeutic and its controlled release, as well as a novel approach for the loading, protection, and release of a neurotrophic factor using heparin-functionalized particles is established. Such particles can be programmed to release antiexcitotoxic therapeutics over a short-time period to potentially reduce the trauma induced by the cochlear implant insertion on hair cells, while releasing neurotrophins over a longer period to protect and preserve the hair cells as well as the SGN in the cochlea to potentially result in a higher level of hearing for patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) Microparticle characterization via zeta potential before and after porosity, B) particle size distribution, and C) SEMs demonstrating various degrees of porosity as a function of the time the particles were immersed in an acidic buffer.

Figure 2.

Microparticle distribution and persistence in the cochlea after 24 hours. Two-photon imaging of the base of the intact cochlea displays the distribution of particles (A-2) and individual images of the different sections of the cochlea (B-1–4) demonstrate that particles persist in the ear after 24 hours and are well distributed in the cochlea. The table contains the quantification of the average number of particles, and their respective percentages, that were found in each turn of the cochlea. The numbers are driven from the average of three sections in the midmodiolar (center) of the cochlea.

Figure 3.

A-1) Schematic of the design of the microparticles, A-2–4) Encapsulation of piribedil in one compartment of microparticles as analyzed by CLSM imaging, and B) its continuous release over a 14 day period.

Figure 4.

Loading and release of GDNF from particles. A) The zeta-potential measurements of each step confirming the presence of the polymers. B-1 and -2), the loading of GDNF, its antibody staining, and the respective confocal images (2D and 3D reconstructions) are displayed. C) The continuous release of GDNF from particles over a 50 day period.

Scheme 1.

Design, fabrication, and processing of bicompartamental particles loaded with two distinct therapeutics.