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A regulated delivery system for inner ear drug application ☆

Shayanne A. Lajud [a,1], Zhao Han [b,1], Fang-Lu Chi [b], Rende Gu [c], Danish A. Nagda [a], Orysia Bezpalko [a], Samudra Sanyal [a], Andres Bur [a], Ziying Han [a], Bert W. O'Malley Jr. [a], Daqing Li [a,*]

[a] Department of Otorhinolaryngology - Head & Neck Surgery, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, United States
[b] Department of Otorhinolaryngology, Eye Ear Nose and Throat Hospital of Fudan University, Shanghai, China
[c] Sound Pharmaceuticals Inc., 4010 Stone Way N., Suite 120, Seattle, WA 98103, United States

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ABSTRACT

Objective: We have recently developed a novel inner ear drug delivery system using chitosan glycerophosphate (CGP) hydrogel loaded with drugs commonly used for treatment of inner ear diseases, significantly improving the drugs' sustained delivery. The goal of this study is to evaluate the effectiveness of chitosanase as a "switch off" mechanism for this drug delivery system when side effects and potential ototoxicities appear during treatment. To evaluate this effect, we tested gentamicin (GENT) in the inner ear following CGP delivery with/without regulation.

Methods: Purified chitosanase was obtained and used for regulating the CGP delivery system. *In vitro* studies were performed to evaluate the effect of the interaction between chitosanase and CGP-hydrogel loaded with GENT or Texas Red-labeled GENT (GTTR). *In vivo* studies were performed using our mouse model to investigate the regulatory effect of chitosanase application on the delivery of GENT to the inner ear. To assess the potential drug rerouting regulatory effect of chitosanase the GTTR fluorescence intensity was evaluated at the round window niche (RWN) and the Eustachian tube (ET). To further characterize this regulatory effect, GENT concentration in the perilymph of the inner ear was analyzed by chromatographic tandem mass spectrometry (LC–MS/MS), and the uptake in the inner ear cells was measured using fluorescence microscopy following CGP delivery with/without chitosanase application.

Results: The chitosanase effectively digested the CGP-hydrogel, quickly releasing GENT and GTTR from the system *in vitro*. When reacted with GENT alone chitosanase did not produce any reducing sugars and did not affect GENT's antimicrobial activity. *In vivo* GTTR was effectively rerouted from the RWN to the ET, limiting its uptake in inner ear hair cells. Concurrent with these findings, GENT concentration in the inner ear perilymph was significantly decreased after chitosanase application.

Conclusion: Our study findings suggest that, for the first time, sustained and controlled inner ear drug delivery can be successfully regulated enhancing its translation potential for clinical application. The use of chitosanase to digest the CGP-hydrogel results in the rerouting of the loaded drug away from the RWN, effectively downregulating its delivery to the inner ear. This important modification to our drug delivery system has the ability to deliver therapy to the inner ear until desired effect is achieved and to stop this process when side effects or treatment-related ototoxicities start to occur, providing a novel and salient approach for safe and effective delivery to the inner ear.

1. Introduction

The management of many inner ear diseases remains a challenge for the otolaryngologist. Systemic therapies to the inner ear are ineffective due to physiological and anatomical barriers that limit access. In addition, systemic delivery is associated with increased risk of systemic side effects and toxicities. Therefore, there is a considerable need for the development of safe and effective local delivery systems that provide sustained and controlled drug release into the inner ear.

Local drug delivery to the inner ear was first described by Schuknecht more than 50 years ago for the treatment of Ménière's disease [1], an idiopathic condition affecting hearing and balance and characterized by episodes of vertigo, tinnitus, and progressive fluctuating hearing loss. In the 1990s, this approach gained popularity when locally applied gentamicin (GENT), *via* an intratympanic (IT) injection, was accepted as an effective treatment for the vertigo component of Ménière's disease [2]. IT GENT primarily enters the inner ear through the round window membrane (RWM), a three layer structure that separates the middle ear cavity from the inner ear perilymphatic fluid, and its kinetics and dynamics are largely determined by the method of delivery [3–5]. It has

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* Corresponding author. Tel.: +1 215 615 0854; fax: +1 215 898 4469.
E-mail address: lidaqing@mail.med.upenn.edu (D. Li).

[1] These authors contributed equally to the work and should be considered co-first authors.

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been shown that GENT causes ototoxicity in a dose-dependent manner [6,7]. Moreover, IT GENT injection shows higher-peak perilymph concentrations and exhibits more variability in all measurements than sustained-release delivery methods [4,8]. Rapid, high-dose perfusion has been found to result in necrotic damage, whereas slow or chronic perfusion has resulted in apoptotic damage [9,10]. Importantly, in clinical trials, patients exposed to titrated regimens of IT GENT experienced less hearing loss and less attenuation of their word recognition, as compared with patients under fixed dose regimens, whereas vertigo control did not seem to be affected by the regimen used [11]. Due to difficulties in objectively determining the drug pharmacokinetics in patients, most treatment regimens have been developed empirically, which may in part explain the large variability in outcomes seen in recent clinical trials [11–14].

Our laboratory has been working on the development of a novel, stable, safe, sustained, and controlled inner ear delivery system that would allow us to effectively achieve the goals of therapy while minimizing iatrogenic morbidity. In previous studies, we have successfully constructed a controlled and sustained local drug delivery system for the treatment of inner ear disease through the delivery of GENT [15] and dexamethasone [16], two of the most commonly used drugs in otologic diseases. The system consisted of a chitosan–glycerophosphate (CGP) hydrogel loaded with the drug that was placed directly on the round window niche (RWN), and at body temperature became an adherent semi-solid gel that persisted in the niche. The porous structure of the CGP-hydrogel allows for the loading of various compounds through direct and indirect molecular interactions. The degradation of the CGP-hydrogel by lysozymes found in the middle ear leads to slow and sustained release of the drug, which diffuses through the RWM into the inner ear's perilymphatic fluid.

Even with a sustained and controlled drug delivery system, treatment regimens often require the ability to be discontinued when symptoms disappear or when adverse side effects first appear. Given the biochemical properties of CGP-hydrogel, our delivery system could be modified to fit these needs through the enzymatic regulation of chitosanase, which is naturally expressed by some microorganisms to specifically digest the chitosan matrix contained in the shells of crustaceous species [17]. Chitosanase degrades chitosan through the endohydrolysis of β-1,4-linkages between D-glucosamine residues, producing reducing sugars and water [18]. Consequently, if CGP-hydrogel is digested by chitosanase, the drug loaded in the system would no longer be in contact with the RWM, and could then be washed away through the Eustachian tube (ET) and into the nasopharynx, effectively limiting its ability to continue to enter the inner ear. The middle ear, where the RWM is located, is covered by a thin mucus layer which lays on top of a ciliated epithelium that beats continuously to clear mucus and any debris from the middle ear to the nasopharynx through the ET [19]. We therefore hypothesized that chitosanase could be used to effectively regulate drug delivery to the inner ear through the degradation of CGP-hydrogel, rerouting the drug away from the RWM through the ET into the nasopharynx (Scheme 1).

In this study, we extracted chitosanase through plasmid expression in *Escherichia coli* and tested its effect on the CGP + GENT-hydrogel delivery system *in vitro*. Additionally, we demonstrated that this enzyme could act as regulator for our CGP + GENT-hydrogel delivery system to the inner ear *in vivo*, through degradation of CGP-hydrogel, release from its adherent position at the RWN and rerouting of the degradation products down the ET, therefore providing an effective system to control the GENT perilymph concentration.

2. Materials and methods

2.1. Plasmids and protein extraction

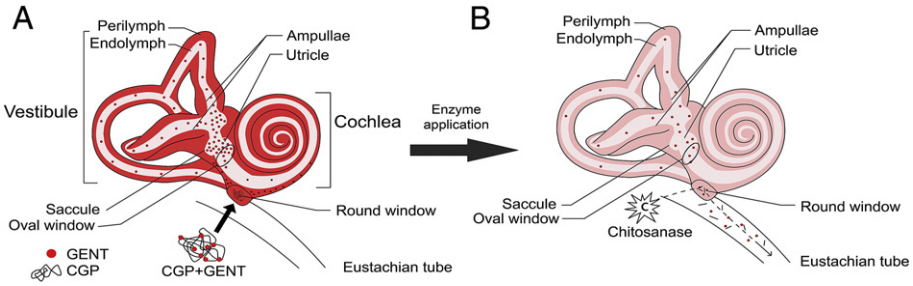
We obtained the plasmid pBMS172 as a gift from Peter Stougaard (Denmark). In this plasmid the chitosanase gene from pET-28a-chitosanase was fused with *E. coli* ompA sequence, substituting the native *Janthiobacterium* signal sequence. In order to purify chitosanase protein, we added a His-tag gene fragment to the N terminal (Fig. 1). We used standard methods to induce the synthesis of protein, followed by its extraction and purification using the Bug Buster protein extraction reagent (EMD Millipore), and a His-bind Purification Kit (Novagen, EMD Millipore), respectively, according to the manufacturer's instructions. Endotoxin levels were evaluated using LAL assay by Cell Center Services, University of Pennsylvania School of Medicine.

2.2. SDS-PAGE analysis and Western blot

To confirm that the target protein was being synthesized we performed a SDS-PAGE gel analysis of the crude protein extract. Briefly, samples were run on a SDS-PAGE gel, followed by staining with Coomassie Brilliant Blue R-250 (Sigma). Purified product was confirmed by Western blot. Polyclonal rabbit anti-His antibody (Cell Signaling, #2365) was used as a primary antibody (1:2000 dilution) and a goat anti-rabbit IgG-HRP (H&L) antibody (Cell Signaling, #7074) was used as a secondary antibody (1:10,000 dilution). MagicMark XP western protein standard (Invitrogen, #LC5602) was used as a molecular weight marker. Gels and films were scanned using a Microtek scanner (Microtek).

2.3. Concentration and in vitro activity of chitosanase

The BCA Protein assay (Thermo Scientific) was used to test the concentration of the purified chitosanase. According to previous reports [17,20], the chitosanase activity is measured by the amount of reducing sugars released during the hydrolysis of colloidal chitosan. The reaction mixture, containing 1 mL of McIlvaine's buffer, 0.5 mL of 2% w/v colloidal chitosan (91.2% DDA, Ultrasan, Biosynthec) and 0.5 mL chitosanase, was incubated at 46 °C for 30 min. The mixture was then boiled for 10 min to stop the reaction. The amount of reducing sugars released in the supernatant was measured by a method that uses 63% dinitrosalicylic acid (DNSA), and the absorbance



Scheme 1. Regulated CGP hydrogel drug delivery system. A) A chitosan–glycerophosphate–gentamicin (CGP–GENT) hydrogel was applied to the RWM, resulting in a steady release of the GENT into the perilymph (represented as red), and a vestibular-dominant distribution (represented as red dots targeted to the hair cells of the ampullae, utricle, and saccule organs of the inner ear). B) Chitosanase was applied after 24 h to stop the GENT delivery, decreasing the levels of the GENT in the perilymph and hair cells of the inner ear (represented as a pale red, and decreased number of dots).