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Label-Free Ferrocene-Loaded Nanocarrier Engineering for *In Vivo* Cochlear Drug Delivery and Imaging

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ABSTRACT

It is hypothesized that ferrocene (FC)-loaded nanocarriers (FC-NCs) are safe label-free contrast agents for cochlear biodistribution study by transmission electron microscopy (TEM). To test this hypothesis, after engineering, the poly(epsilon-caprolactone)/polyglycolide NCs are tested for stability with various types and ratios of sugar cryoprotectants during freeze-drying. Their physicochemical properties are characterized by UV-visible spectroscopy, dynamic light scattering, Fourier transform infrared spectroscopy, and scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM/EDS). The biodistribution of the FC-NCs in the cochlear tissue after intratympanic injection in guinea pigs is visualized by TEM. Auditory brainstem responses are measured before and after 4-day treatments. These FC-NCs have 153.4 ± 8.7 nm, 85.5 ± 11.2%, and -22.1 ± 1.1 mV as mean diameters, percent drug association efficiency, and zeta potential, respectively ($n = 3$). The incorporation of FC into the NCs is confirmed by Fourier transform infrared spectroscopy and SEM/EDS spectra. Lactose (3:1 ratio, v/v) is the most effective stabilizer after a 12-day study. The administered NCs are visible by TEM in the scala media cells of the cochlea. Based on auditory brainstem response data, FC-NCs do not adversely affect hearing. Considering the electrondense, radioactive, and magnetic properties of iron inside FC, FC-NCs are promising nanotemplate for future inner ear theranostics.

Introduction

Cochlear drug delivery remains one of the emerging challenges in the area of drug delivery systems. Currently, there is a critical and urgent need for an improved method to explore the delivery and the biodistribution of drug-loaded nanoparticulate systems in the inner ear. Several delivery systems, including microparticles, ultrafine fibers, nanosponges, liposomes, microcatheter, hydrogels, and nanoparticles, have been proposed for cochlear drug targeting and biodistribution, diagnostics, and imaging.[1-5] Lately, the use of nanoparticulate systems has been of much interest for the delivery of drugs and contrast agents in cochlear tissue.[6] Our recent studies have shown that polymeric nanocarriers (NCs), made of poly-(lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone)—poly(ethylene glycol) (PCL—PEG) diblock can be a suitable platform for drug delivery into the cochlea.[7] Moreover, we have shown that NCs could be taken up by two types of cochlear cell lines, namely HEI-OC1 and SVK-1 cells.[8] Various substances, such as fluorophore, iron oxide, magnetic, superparamagnetic, or Technetium 99m—loaded NCs, have been used to study the particle distribution in the cochlea after a round window membrane (RWM) injection through the intratympanic route.[6,9,10]

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However, the toxicity of nanoparticles has raised some human and environmental concerns.[11,12] More specifically, direct drug delivery into the cochlea has been associated with the risk of irreversible damage of the cochlear tissue, thus impairing the hearing function.[13] Recently, an *in vivo* study highlighted the use of magnetic force to pull payloads on NCs into the inner ear in guinea pigs with the risk of exceeding the US Food and Drug Administration safety limits.[10] Therefore, there is a need to engineer a relatively simple nanoparticulate system that does not cause hearing loss toward the goal of exploring the biodistribution and safety of nanoparticles in the cochlear tissue. In other words, the development of a reliable contrast agent to investigate the biodistribution of drug carriers in the cochlea without reducing the hearing ability or facing some regulatory issues is an urgent need. Several techniques, such as fluorescent microscopy[14] or electron microscopy, have been proposed to investigate the cochlear distribution of nanosized particles. Over the years, electron microscopy has become a vital tool for direct characterization and imaging of nanomaterial in biological specimens.[15,16] An electron microscopy image is generated by the differential electron scattering between the biological material and a surrounding heavy-metal stain layer, generating a high contrast image of specimen. Heavy metal conjugates, such as methylamine tungstate, silicon tungstate, uranyl acetate, uranyl nitrate, and phosphotungstic acid, have been chosen for their strong electron scattering properties.[17] Conventional electron microscopy technique of a negatively stained biological specimen has the disadvantage of being laborious for the detection of nanoparticles whether or not they contain a contrast-enhancing agent.[18] Bearing that in mind, it becomes a necessity to conduct an investigation into biological tissues using contrast-enhancing agent—loaded nanoparticles and TEM without negative staining.

Ferrocene (FC) is a stable organometallic compound (Mw, 186.03 Da) that was discovered in 1951.[19] It consists of 2 cyclopentadienyl rings bound on opposite sides of a central iron (Fe) atom in a sandwich-like fashion[20,21] (Supplementary Fig. S1). A total of 12 electrons (6 from each ring) are made available to the iron via covalent bonding. Moreover, the electron density ($|\Psi(0)|2$) at the Fe nucleus for FC is 11,335.010, with an ionicity of +1.39.[22] The oxidation and reduction potentials of FC are 0.508 V and 0.421 V, respectively.[23] FC-based compounds continue to gain potential interest because of recent findings in various research fields such as chemistry, biology, and nanomedicine.[24-27] FC has been used for the synthesis of nanoparticles, functionalized carbon nanotubes, and polymerosomes.[26-28] To our knowledge, it has never been encapsulated in NCs for hearing research purpose.

Biodegradable polymers such as PCL, PEG, poly(lactic acid), and PLGA have been among the most attractive polymeric candidates used for drug delivery.[29,30] PCL forms a hydrophobic core that can potentially allow a high association efficiency of hydrophobic drugs through hydrophobic interactions. PEG forms the hydrophilic protective shell of the NCs and has been found to be biocompatible and resistant to both protein adsorption and cell adhesion.[31] In this study, FC-NCs were intended to be probed for imaging and cochlear biodistribution studies without the magnetic force usually involved in magnetic resonance imaging (MRI). It was hypothesized that FC-NCs can be a potential contrast agent to investigate the cochlear biodistribution using TEM analysis without additional staining or reducing the hearing ability *in vivo*. To minimize the aggregation of the NCs and to facilitate the particle redispersion before administration, 3 well-known cryoprotectants were investigated during the freeze-drying process. The morphologic analysis of FC-NCs was carried out by EM. Furthermore, the ultrastructural analysis of the NCs' biodistribution in the cochlea, and the effect of the FC-NCs on hearing ability were tested using TEM and auditory brainstem response (ABR) techniques, respectively.

Experimental Procedure

Materials

FC, sucrose, acetone, and dichloromethane were obtained from Sigma-Aldrich (St. Louis, MO). PLGA with a L/G ratio of 50:50 and inherent viscosity of 0.39 dL/g was purchased from Birmingham Polymers (Pelham, AL). Poly(ε-caprolactone)-poly(ethylene glycol) diblock (PCL-b-PEG [3 kDa-2 kDa]) was provided by Advanced Polymer Materials Inc. (Montréal, Canada). Alpha lactose and hexane were purchased from Fisher Scientific Inc. (Pittsburgh, PA). D-mannitol was obtained from Acros Organics (Morris Plains, NJ). All other chemicals used in the study were either analytical grade and used without further purification or EM grade.

Synthesis of FC-Loaded NCs

FC-NCs were prepared by solvent-diffusion method adapted from previous work.[32] The NCs were formulated by varying the amounts of FC, PLGA, and PCL—PEG. Briefly, polymers were dissolved in 1 mL of acetone (organic phase) containing an appropriate amount of FC. The resulting solution was hereby added dropwise to an aqueous phase (20 mL) without surfactant, under constant stirring at 1100 rpm (stirrer model RO 15; IKA-Werke Gmbh & Co, Staufen, Germany). The organic phase was evaporated at 25°C under constant stirring for 2 h. Then, the FC-NCs were isolated by centrifugation at 15,000 rpm for 20 min at 8°C (VWR International Micro 18R, Darmstadt, Germany), and resuspended in appropriate sugar solution as described below (Direct-Q 3 UV system; Millipore SAS, Molsheim, France). The colloidal suspension was frozen at −196°C in liquid nitrogen (N_2) and dried at −47°C, 0.01 mBar for 12 h (Freezone 1; Labconco Corp., Kansas City, MO). Blank NCs were similarly prepared without FC.

Analysis of Particle Mean Diameter, Polydispersity, and Zeta Potential

The powdered FC-NCs (2 mg) were suspended in 1 mL of phosphate-buffered saline solution (PBS, pH = 7). Then, 100 μL of the colloidal suspension was withdrawn and diluted 10 times in PBS. The resulting suspension was analyzed for particle mean diameter (PMD), polydispersity index (PDI), and zeta-potential (ζ) at 25°C using dynamic light scattering method (DLS, Zetasizer Nano ZS; Malvern Instruments Ltd, Worcestershire, UK). In accordance to the National Institute of Standards and Technology, samples with a PDI <0.05 were considered monodisperse and otherwise polydisperse.[33]

Percent Drug Association Efficiency of FC-Loaded NCs

The percent drug association efficiency (AE%) of FC in the NCs was determined according to a previous method.[7] Freeze-dried FC-NCs (3 mg) were dissolved in 0.1 mL of dichloromethane, sonicated for 10 min, and diluted in hexane at the ratio of 1:14 v/v. The colloidal suspension was centrifuged at 15,000 rpm for 20 min at 4°C. The amount of FC was determined from the supernatant at 325 nm using a Spectronic Genesys 10 Bio UV-visible spectrophotometer (Thermo Fisher Scientific Inc., Pittsburgh, PA). The amount of FC was determined based on the absorbance of the nanoformulation extract and considering the FC calibration curve