Feasibility of Superparamagnetic Nanoparticles for Drug Delivery to the Inner Ear


*University of Oklahoma HSC, Department of Physiology
940 S.L. Young Blvd., Oklahoma City, OK, USA ken-dormer@ouhsc.edu
**Hough Ear Institute, Oklahoma City, OK, USA rickkopke@swbell.net, liujx@integris-health.com, chenkx@integris-health.com
*** NanoBioMagnetics, Edmond, OK, USA dgibson@nanobmi.com
**** Naval Medical Center-San Diego, CA, USA rjackson@nmcsd.med.navy.mil, mcostello@nmcsd.med.navy.mil
*****University of Oklahoma, Chemical, Biological & Materials Engineering, Norman, OK USA fadee-mondalek@ouhsc.edu

ABSTRACT

The inner ear is difficult to treat with therapeutic substances because of blood supply limitations and the transducing and supporting cells being bathed in inaccessible perilymphatic and endolymphatic fluids. The purpose of this study was to determine the feasibility of using external magnetic fields to pull superparamagnetic nanoparticles with an associated gene across the Round Window Membrane (RWM) and into the perilymph. Silica-encapsulated magnetite nanoparticles (Si-MNP; 30 nm diameter) were synthesized, characterized and tested for biocompatibility in a non-dividing cell model and in organotypic (three day mouse pup) Organ of Corti (OC) cultures. Particles were internalized without magnetic attraction and cells were indistinguishable from control cells. The MATH-1 gene [1] was inserted into a plasmid with promoters to facilitate intracochlear transfection and this plasmid was tested for viability in a non-dividing cell line. Studies are ongoing to combine the plasmid with Si-MNP in a carrier nanoparticle for RWM transport and release of plasmid in the perilymph.

Keywords: cochlea, MATH-1, magnetic, transfection, ear

1 NANOPARTICLE SYNTHESIS

We sought to synthesize a nanoparticle that could be pulled into the inner ear by an external magnetic field. Superparamagnetic, silica encapsulated magnetite nanoparticles (Si-MNP) with diameters of ~ 30 nm were prepared by initially synthesizing magnetite according to the procedure of Massart [2]. The nanoparticles were coated with silica according to the method of Correa-Duarte [3].

1.1 Electron Spin Resonance  In order to test for potential exposure of Fe to cells, aqueous 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was reacted with activated charcoal and then filtered. To disclose reactive iron surfaces a Fenton type reaction was performed with 10 mM H2O2 added to a solution containing the DMPO spin trap and the SI-MNP’s. Fig 1 shows a spectrum investigating the interaction of SI-MNP’s in a Fenton system. Spectrum A in each of these figures represent the DMPO spin trap with 10 mM H2O2, Spectrum B is the addition of the SI-MNPs to the DMPO + H2O2 reaction system of spectrum A. Spectrum C is a positive control of adding 60 µL of ferrous sulfate to the reaction seen in spectrum B. The ESR spectrometer settings used in all experiments were: microwave power, 1 mW; microwave frequency, ≈9.850173; modulation, 1 Gauss; centerfield, 3510 Gauss; sweep width, 100 Gauss; gain, 2 x 104. As can be seen in the following spectra, there is no evidence of free radical activity, supporting the Si encapsulation of the nanoparticles without exposed iron available for generation of free radicals.

![Fig 1. ESR spectra showing the non-detection of free iron in the silica encapsulated Fe3O4 nanoparticles](image-url)
A tissue culture model of the Round Window Membrane (RWM) of the cochlea was made using small intestine submucosa (SIS), seeded with fibroblasts and monolayer of urothelial cells on the SIS matrix [4]. The culture assembly was placed in 24 well culture dishes until the urothelium layer was confluent. Solutions of magnetite nanoparticles (MNP) in saline, water or culture medium were placed on the membrane and subjected to an attracting magnetic force from NdFeBo magnets placed directly under the 24 wells of the culture plate. MNP, 30-130 nm diameter, were pulled through 2 cell layers of the in vitro model. MNP were synthesized by NanoBioMagnetics™ Inc. (Edmond, OK) or Micromod™ (Rostock, Germany). Results described are for (Nanomag-D NH2) dextran coated agglomerates, 130 nm diameter, each experimental agglomerate consisting of multiple MNP with diameters of 10 nm to 20 nm. The magnetic exposure (0.3 T) to the culture and particles lasted 60 minutes. Afterwards, cells were fixed with 4% paraformaldehyde and sectioned for histology. The effluent solution of NPs at the bottom of the insert well was collected and imaged using Transmission Electron Microscopy (TEM) to verify NPs had traversed the membrane. Preliminary experiments conducted on 3 culture inserts all showed the ability of the magnetic delivery system to pull MNP through the RWM model. The SIS membrane-urothelium tissue model of the RWM is shown in a histological section in Fig 2.

RWM in fresh frozen human temporal bones also were subjected to 30 nm diameter Si-MNP placed in the RW niche after surgically exposing the cochlear membranes. The Si-MNP were exposed to external magnetic field of 0.3 Tesla for 3 X 20 min. The perilymph was carefully aspirated and presence of Si-MNP was proven using transmission electron microscopy with electron energy loss spectroscopy (TEM with EELS).

### 3  RAT AND GUINEA PIG ROUND WINDOW MEMBRANE TRANSPORT

Magnetic transport of MNP through the live RWM was proven in both adult Sprague-Dawley rats and guinea pigs. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg, i.m). The tympanic bulla was opened, the RWM niche exposed and approximately 1 ul of 1 mg/ml aqueous solution placed on the RWM surface. The anesthetized rat was then positioned 2.5 cm on the surface of a 10 cm cuboidal NdFeBo magnet (0.3Tesla) so that the RWM was parallel to the surface of the magnet. The magnetic field was present for 20 minutes then the solution changed on the RWM surface and the exposure repeated (total exposure time was 60 min.) The control rats had the same treatment, but without magnetic exposure. A small hole was then placed in the apex of the cochlea and the perilymph aspirated using a 30½ Ga needle and examined using TEM. The temporal bones of the rat were removed and fixed in 4% paraformaldehyde and 1 % glutaraldehyde in 0.1 M PBS. Perilymph samples from 3 experimental rats exposed to magnetic field during experiment all showed nanoparticles in the perilymph. Perilymph from control rats is still being processed.

### 4  BIOCOMPATIBILITY OF AM-SI-MNP

Potential toxicity of an alloplastic material such as Si-MNP in the inner ear is a vital concern for implants and so tissue response to Si-MNP was tested on organotypic cell cultures of the Organ of Corti (OC) from 3 day mouse pups. Aminated Si-MNP were washed with deionized water 3-5 times then soaked in 70% ETOH for 1-2 days, and oven dried. Twenty mg of particles were added to 2ml deionized water (stock solution). The experimental solution (100µg/ml) was made by diluting 2 ml of culture medium.

**4.1 Application to Organotypic cultures**

Cultures of OC were maintained on collagen gel in 35mm plates for 24 hrs in BME (Basal Medium Eagle) media at 37 degrees C, room air with 5% CO. Aminated Si-MNP (AmSiMNP) were added to cultures of experimental tissues. After 48 hrs. the cultures were washed and fresh medium with AmSiMNP replaced. Organotypic cultures without nanoparticles served as controls, maintained under the same conditions. After 2 and 3 days cultures were fixed in 4% paraformaldehyde and stained with FITC-phallodin. Tissues were mounted on glass slides with Vecastain and examined under a fluorescent microscope.

These preliminary observations, at the light microscopic level shown in Fig 3, reveal no hair cell damage or loss associated with the presence of AmSiMNP. There was no observable toxicity to the hair cells at concentrations as high as 100 µg/ml. Micrographs A & B are from control.
tissues, while C & D cultures were exposed to AmSiMNP for 2-3 days.

Fig 3. Hair cells from mouse pups. Controls A, B; Experimental cultures C, D.

5 MATH-1 PLASMID DESIGN AND TRANSFECTION

MATH-1 is the basic helix-loop-helix (bHLH) transcriptional factor and is a positive regulator of cochlear hair cell differentiation. First time ever, MATH-1 has been shown to restore hearing in a mammal [1]. A custom vector was constructed (Fig 4) on the bases of the pIRES2-EGFP vector (Clontech) and the coding sequence of MATH-1 inserted into the multiple cloning site (MCS). The plasmid contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (EGFP) coding region. This vector also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen to assist in nuclear membrane penetration in non-dividing cells.

To evaluate the viability of the custom plasmid with the MATH-1 insert, lipofectamine protocol was used for transfection of human kidney cells. The original vector without any inserts was used as a negative control. After 48 hours the transfected cells were lysed and the lysis analyzed for MATH-1 protein using Western Blot analysis. MATH-1 protein was expressed.

Subsequent studies were performed using the cell membrane penetrating peptide TAT (Trans-Activating Transduction protein) added to the plasmid. We sought to transfect the plasmid in a non-dividing cell line (ARPE-19, human retinal epithelial cells). Plasmid (pGL3 luciferase containing) was used as control and was also complexed with TAT peptide (0.01 mg/ml). Luciferase assay showed signal expressed protein of 30-50, greater than the plasmid alone. Future studies will incorporate TAT into a polymer carrier of both plasmid and nanoparticles.

Plasmids containing the CMV promotor (phMGFP) for green fluorescent protein and the SV40 promotor (SV-4—GFP and pIRES-MATH-1) were microinjected into confluent non-dividing human ARPE-19 cells. Cytoplasmic and nuclear injections of plasmid construct with SV40 promotor resulted in expression of GFP in these non-dividing cells (modeling the supporting cells of the Organ of Corti).

Fig 5 shows the results of CMV promotor microinjection where green color = green fluorescent protein. Red = indicator showing cytoplasmic vs nuclear injections. Yellow = expression of GFP. Only nuclear injection resulted in GFP expression. The green color in the cytoplasm resulted from nuclear over-expression and leakage into the cytoplasm. No injections in cytoplasm resulted in nuclear expression of GFP.

Fig 6 shows the results of cytoplasmic microinjection of plasmid with SV40 promoter, there was GFP expression by the nucleus. This demonstrated that the specially designed plasmid with the SV-40 promoter enhancer facilitated...
transport of the plasmid through the nuclear membrane where the expression of GFP occurred.

6 SUMMARY AND FUTURE DIRECTIONS

The goal of these parallel studies has been to design and test an inorganic delivery system for therapeutic substances to the inner ear in humans. We have fabricated nanoparticles that have relatively high susceptibility to an external magnetic field. Nanoparticles (Fe3O4) have been pulled through an epithelial membrane model, the ex vivo human and the in vivo rat and guinea pig RWM. The particles were compatible with cell cultures of non-dividing ARPE-19 cells and with organotypic cultures of mouse Organ of Corti. The particles did not promote apoptosis. One delivery substance for our magnetic targeting system is the MATH-1 gene. We have incorporated MATH-1 into a plasmid along with SV40 for nuclear targeting. TAT was tested in non-dividing cells to promote membrane penetration, a particular hurdle for a non-viral vector in the cochlear supporting cells. MATH-1 protein expression by the custom plasmid has been demonstrated using Western Blot. Finally, the plasmid has been microinjected into the cytoplasm of ARPE-19 cells and the SV40 successfully expressed GFP.

Future experiments will evaluate the combination of plasmid with its promoters, the nanoparticle and a polymer carrier matrix. The ultimate goal will be to transport the carrier system across the RWM, where the nanoparticles will be released and the polymer with TAT will facilitate cell membrane penetration into supporting cells, wherein the SV40 will facilitate nuclear delivery of the plasmid containing MATH-1.

Acknowledgement: We deeply appreciate the assistance of Eric Howard, PhD, Department of Cell & Molecular Biology, for assistance in plasmid construction and Yuanyuan Zhang, MD, PhD, Department of Urology, for guidance in culture of urothelial-SIS membranes, both from University of Oklahoma Health Sciences Center.

Disclosure: K. Dormer is an Officer of NanoBioMagnetics Inc.

REFERENCES


