Internalization of Nanoparticles in the Middle Ear Epithelium in Response to an External Magnetic Field: Generating a Force.

K. Dormer[*], C. Seeney[**], A. Mamedov[***] and F. Mondalek[****]

[*]University of Oklahoma Health Sciences Center, Department of Physiology Room 634, 940 Stanton L. Young Blvd., Oklahoma City, OK, USA, kenneth-dormer@ouhsc.edu
[**]NanoBioMagnetics INC., 124 N. Bryant Ave. Edmond, OK, nanobio@att.net
[***]Nomadics, Inc., 1024 S. Innovation Way, Stillwater, OK, amamedov@nomadics.com
[***] University of Oklahoma, Department of Chemical Engineering and Materials Science 100 E. Boyd, Room T-335 Norman, OK, fadee@ou.edu

ABSTRACT

In this study we report the first generation of force by long-term intracellular magnetic nanoparticles (MNP) using a guinea pig middle ear model. Synthesis of magnetite nanoparticles was done using a modified precipitation technique. We used different methods to characterize MNP. The mechanism for internalizing 16 ± 2.3 nm diameter, silica coated, superparamagnetic, MNP was likely magnetically enhanced endocytosis. During the surgery a rare earth, permanent magnet (0.35 Tesla) placed under the animal was used to pull the MNP into the tissue. After 8 days, following euthanasia, tissues were harvested and confocal scanning laser interferometry used to verify intracellular MNP. Displacements of the osscicular chain in response to an external sinusoidal electromagnetic field were measured. Laser Doppler interferometry, measured, for the first time, a normal biomechanical function enabled by intracellular MNP.

Keywords: *magnetic nanoparticles, ear, hearing, superparamagnetic, intracellular delivery.*

MATERIALS AND METHODS

Spherical particles of magnetite (Fe_3O_4) included an external shell of silica (SiO_2) and were 10-20 nm in diameter were synthesized in collaborative effort of NanoBioMagnetics Inc. (NBMI, Edmond, OK) and Nomadics, Inc. (Stillwater, OK). Obtained core-shell MNP then has been treated to form surface amine groups, which provide attachment sites to other molecules. MNP were conjugated with fluorochrome fluoroscein isothiocyanate (FITC) using the amine linkers, following standard conjugation protocol (Molecular Probes, 2003). After the conjugation, several rinses were made using sterile 0.9% saline and the particles were stored in saline suspension at 20 ^{o} C until used.

1.1 MNP Synthesis. Magnetite nanoparticles were synthesized using a modified procedure of Massart (Massart 1981; Philipse 1994). A 2 M iron (II) sulfate heptahydrate solution was prepared in 2 M HCl and combined with 1 M iron (III) chloride hexahydrate aqueous solution. The solutions were mixed and added to 0.7 M ammonium hydroxide with rapid stirring. The resulting gelatinous precipitate was stirred for 30 minutes then the precipitate was collected using a small magnet with removal of the supernatant. After several such washes, the precipitate was resuspended in 0.7 M ammonium hydroxide, and peptized through the addition of 1 M tetramethylammonium hydroxide aliquots. The total volume of this suspension was then taken to 250 ml.

1.2 Silica Coating. The magnetite particles were then coated with silica according procedure described elsewhere (Correa-Duarte 1998). Briefly, a suspension of magnetite nanoparticles was vigorously stirred and a 4 ml aliquot taken up to a volume of 100 ml with distilled water. A solution of 0.54% sodium silicate was prepared at pH 10.5, and 4 ml was added to the previously prepared magnetite nanoparticles suspension. The suspension pH was adjusted to 10.0 and stirred for 2 hours and then allowed to stand for 4 days, after which the excess silica was removed by washing several times with distilled water by the aid of a magnet.

1.3 Amine Linkers. To make the silica coated core-shell MNP biocompatible and suitable for our biological application, amine groups were attached to the surface. Particles were treated with 3-aminopropyl trimethoxy silane then a 1 ml aliquot of

the suspension was brought to a volume of 5 ml with distilled water and sufficient 3-aminopropyl trimethoxy silane added to give a final concentration of 5% (Wang 2001). The reaction system was occasionally stirred at room temperature for an hour. After the incubation period, the particles were washed with distilled water with the aid of a magnet. The Kaiser assay was next used to confirm attachment of functional group onto the surface of the silica coated nanoparticles (Kaiser 1970).

1.4 MNP Characterization The MNP crystal structure, morphology, size distribution, elemental and chemical composition were investigated using powder x-ray diffractometry (XRD) (Scintag X'TRA), transmission electron microscopy (TEM), and energy dispersive spectroscopy (EDS). Crystal structure (phase) and average MNP size found in a macroscopic sample can be determined using XRD techniques. For this work, crystallinity, phase and crystal size are important properties because they affect the magnetic susceptibility of the particles. TEM analysis allows individual particles to be directly imaged (Figure 1). These images are used to determine the specific morphology, chemical composition, as well as size and size distribution of MNP. High-resolution TEM (HRTEM) and electron diffraction can show whether an individual MNP is a single crystal or poly-crystalline. Of importance here, different materials

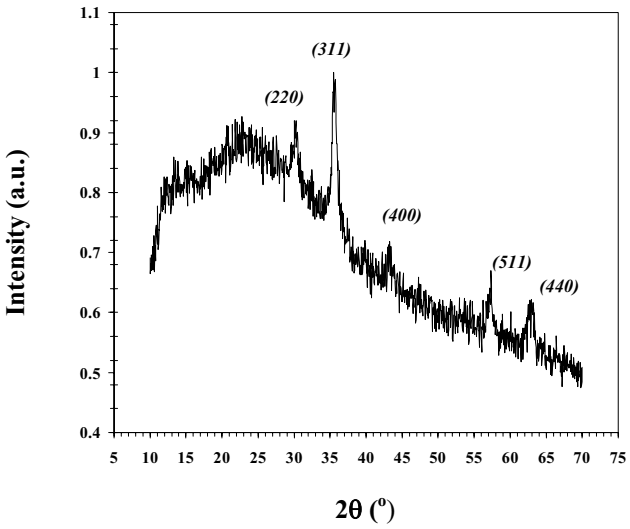

Figure 2 XRD pattern of uncoated iron oxide MNPs, indexing corresponds to Fe3O4 magnetite.

such SiO_2 (silica coating) iron oxide (magnetic core) can be differentiated even on single particles, indirectly through electron diffraction and directly through Z-contrast imaging. Thus under the appropriate imaging conditions silica-coated iron-oxide NPs can be differentiated from non-coated NPs. The elemental constituents can also be unambiguously determined using EDS (typically with resolution larger than the electron beam size itself). In EDS analysis the electron beam interacts with the sample and excites atomic core-shell (*e.g.* K-shell) x-rays. The peaks in the collected x-ray spectra are characteristic of the elemental constituents of the nanoparticles. The X-ray diffraction spectra (Figure 2) were taken from 10° to 70° (2θ values) using Cu Kα radiation. TEM experiments, including selected area electron diffraction (SAED), HRTEM and EDS, were all performed on a JEOL 2000FX operated at 200 kV equipped with a KEVEX EDS system. Initial assessment of the nature and extent of silica surface treatments was tested. Particle samples that ranged in silica content, based on reaction feed

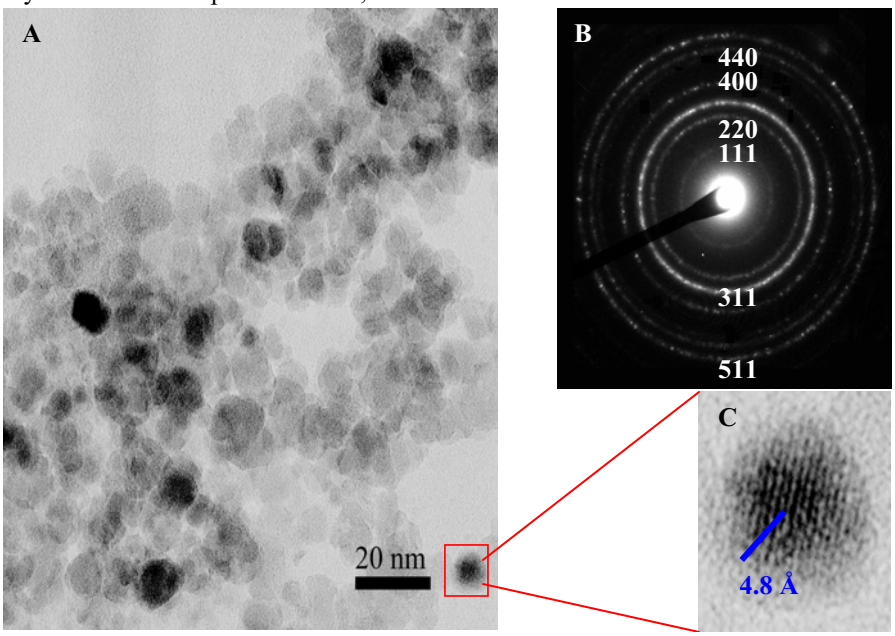

Figure 1 (A) TEM image of uncoated magnetite MNP. (B) SAED pattern from large area of NPs. (C) Zoom-in showing HREM image of an individual MNP as marked in (A).

ratio and time conditions, were dispersed in 0.9% saline and the settling rates were observed as a function of time. Particles with higher ratios in the reaction feed will have the longest settling rates. Hermeticity of the silica coating and nascent resistance to corrosion of uncoated magnetite nanoparticles was tested by soaking both uncoated and silica-coated nanoparticles in 10% NaCl solution for 2 days at room temperature and then 2 days at 40^0C (total of 4 days). The presence of Fe ions was assayed periodically during the 48 hrs. using a standard sodium thiocyanate (NaSCN) test. Long-term tests for corrosion, up to 6 months, were also performed on the stock solution of the MNP, an unprecipitated ferrofluid in tetramethyl ammonium hydroxide. Additionally, precipitated MNP that were placed in aqueous solution, double distilled water at pH = 13, were tested after 5 months of soaking using the sodium thiocyanate test for the Prussian Blue Reaction.

1.5 Cellular Uptake. The MNP, suspended in sterile physiological saline at pH = 7.4 to a flowing paste consistency, were sonicated (Sonicor, Copaigue, NY) for 2-3 minutes before placement pigs. Sonication was used to resuspend particles before placement onto tissues. Next, a 25 µl drop of the MNP suspension was delivered to the epithelial surface whereupon the drop flowed onto the target tissue. This was repeated for an approximate application volume of 50-75 µl, essentially bathing the incus or tympanic membrane with the suspension. During recovery from anesthesia each animal was placed onto the pole face of a 4 x 4 x 4 in. permanent magnet (NdFeBo, 50 MGO) while maintaining the surgical position of experimental ear facing upward. Thus, the upward surface of the incus or tympanic membrane with MNP held by surface tension, then exposed to a magnetic field pulling the MNP downward and into the epithelia. The experimental incus and tympanic membrane at distances of 1 in. from the pole face of the magnet were exposed to a magnetic field of approximately 0.35 Tesla. Each animal was exposed to this external magnetic field for 20-30 minutes, during recovery from anesthesia. They were subsequently returned to their cages for 1-15 days of monitored survival.

1.6 Histology. The experimental incus from the middle ear of each guinea pig was surgically removed and placed in 80% ethanol, 10% normal saline for a minimum of 24 hours. Next the incii were decalcified (Decalcification Solution™, Richard Allen Scientific, Kalamazoo, MI) for 3 days and subsequently automatically processed for paraffin sectioning (Tissue-Tek VIP™, Sakura Finetek, Torrance, CA) with the exclusion of aldehydes to prevent autofluorescence. Transverse 5 µm microtome sections of the incii or tympanic membrane in paraffin blocks were mounted and either stained using hematoxylin and eosin for histopathology or unstained for confocal laser microscopy. Unstained transverse sections were examined using an Argon laser scanning Spectral Confocal and Multiphoton Microscope (Leica Model TCS SP2, Leica Microsystems, Mannheim, Germany). Stacks of 10-20 scans were made through the epithelium lining the incii, or the tympanic membrane epithelium while looking for intracellular fluorescence, indicating the presence of MNP (Figure 3).

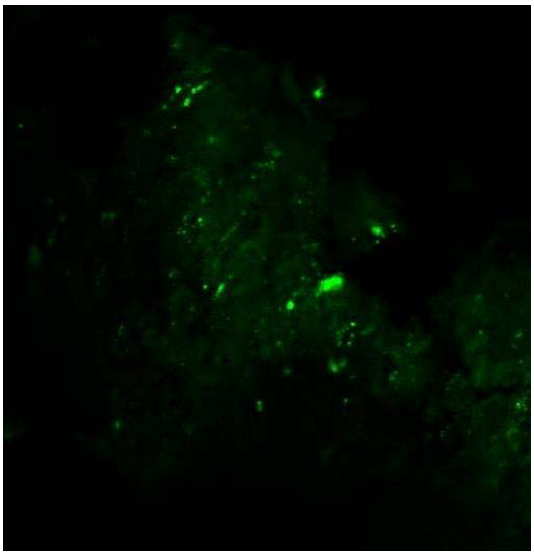

Figure 3 100X confocal micrograph of guinea pig tympanic membrane implanted for 8 days with FITC-labeled MNP

1.7 Laser Doppler interferometry. Biomechanical confirmation of tissue implantation was made using laser interferometry. Immediately following euthanasia, an animal was placed in a lateral recumbent position with the implanted ear up. The pinna was removed so as to provide access to the bony ear canal and operating microscope visualization of the tympanic membrane. Next, a 1 x 1 mm piece of reflective tape (3-M, Minneapolis, MN) was placed near the umbo of the tympanic membrane and displacements measured using a single point, helium neon laser Doppler interferometer (LDI, Model OFV 501 and Model 3000 Controller, Polytec PI, Tustin, CA) caused by an external magnetic field interacting with the implanted MNP. The LDI provided contactless

velocity information with a frequency range of 0-150 Hz, with a fringe counter converting velocity into displacement (Gan 1997). The electromagnetic coil from the *Integrated Sound Processor* of a semi-implantable hearing device (SOUNDTEC, *direct system*™, Oklahoma City, OK) was positioned 1-2 mm from the surface of the tympanic membrane implanted with MNP (Hough 2001). When the coil was activated with a 1000 Hz sine wave at 5-8 volts, peak to peak (Model 80 Function Generator, Wavtek, San Diego, CA and Model 2706 Precision Amplifier, Bruel & Kjaer, Denmark) the interferometer recorded acceleration and calcuated displacements. In two of the animals, interferometry was used to measure displacements of implanted incii. The surgical wound was exposed, accessing the middle ear cavity, and the tip of the electromagnetic coil placed 1-2 mm from the surface of the implanted incus. The same electromagnetic signal was presented and displacements recorded from reflective tape on the lateral surface of the incus. The ossicular chain and tympanic membrane were in tact in all three of these animals; therefore the displacements recorded were movements of the whole middle ear, ossicular chain.

CONCLUSION

We have shown that silica encapsulated, magnetite nanoparticles can be used in conjunction with an external magnetic field to produce a biomechanical force in tissues. MNP, customized for ease of internalization and long-term biocompatibility, can be implanted in epithelia without producing necrosis or apoptosis and remain viable for at least 8 days. Auditory frequencies (subthreshold) have been generated in the in tact ossicular chain of the guinea pig using an electromagnetic coil of the type used in implantable hearing devices.

AKNOWLEDGEMENTS

We gratefully recognize Kevin Lewelling, Ph.D., Guoda Lian, Ph.D., and Matthew Johnson, Ph.D. Department of Physics & Astronomy, Oklahoma University, for their characterization of the nanoparticles using electron microscopy and X-ray diffractometry.

REFERENCES

[1] Aliev, F. G., Correa-Duarte, M.A., Mamedov, A., Ostrander, J.W., Giersig, M., Liz-Martin, L.M., Kotov, N.A. (1999). "Layer-by-layer assembly of core-shell magnetite nanoparticles; Effect of silica coating on interparticle interactions and magnetic properties." Adv. Materials **11**(12): 1006-1010.

[2] Austin, D. F. (1994). "Acoustic mechanisms in middle ear sound transfer." Otolaryngologic Clin N America **27**(4): 641-654.

[3] Correa-Duarte, M. A., Giersig, M., Kotov, N. A., and Liz-Marzan, L. M. Langmuir **14**: 6430-6435, 1998.

[4] Gan, R. Z., Wood, M.W., Ball, G. R., Dietz, T.G., Dormer, K.J. "Implantable hearing device performance measured by laser Doppler interferometry." Ear, Nose & Throat J **75**(5): 297-309, 1997.

[5] Hough, J. V. D., Dyer Jr., R.K., Matthews, P., Wood, M.W. "Early clinical results: SOUNDTEC implantable hearing device phase II study." Laryngoscope **111**: 1-8, 2001.

[6] Kaiser, E. T., Colescott, R. L., Bossinger, C. D., and Cook, P. I. Analytical Biochem **34**: 595-598, 1970.

[7] Mamedov, A., Kotov. N.A. (2000). "Free-standing layer by layer assembled films of magnetite nanoparticles." Langmuir **16**: 5530-5533.

[8] Massart, R. "Preparation of aqueous magnetic liquids in alkaline and acidic media." Transactions on Magnetics **Mag-17**(2): 1247-1249, 1981.

[9] Philipse, A. P., Van Bruggen, M. P. B., and Pathmamanoharan, C. "Magnetic silica dispersions: preparation and stability of surface modified silica particles with a magnetic core." Langmuir **10**(1): 92-99, 1994.

[10] Wang, N., Naruse, K., Stamenovic, D., Fredberg, J.J., Mijailovich S.M., Tolic-Norrelykke, I.M., Polte, T., Mannix, R., Ingber, D.E. "Mechanical behavior in living cells consistent with the tensegrity model." Proc Natl Acad Sci **98**: 7765-7770, 2001.