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# Water-soluble iron oxide nanoparticles for nanomedicine

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## Background/Significance

Monodisperse iron oxide nanoparticles (MION) are easily synthesized in organic solvents for industrial applications. However, biological applications require that the particles be readily dispersed in aqueous solutions. To improve their dispersion in aqueous solution, MION particles can be conjugated to water soluble polymers. These water soluble particles can then be used for nanomedicine, which utilizes nanometer scale constructs to treat diseases at the cellular level. Here we report the synthesis of a monodisperse iron oxide nanoparticle construct for breast cancer applications.

## Materials and Methods

### Chemical synthesis

#### Monodisperse iron oxide nanoparticle (MION) synthesis

All nanoparticle synthesis reagents were purchased from Sigma-Aldrich (St. Louis, MO). Chloroform and acetone were obtained from Mallinckrodt Baker (Phillipsburg, NJ). MION particles were synthesized using oleic acid as a surfactant to keep the particles from aggregating (Figure 1). 2mmols iron oxide hydroxide FeOOH, 10mmols oleic acid, and 10.0g 1-octadecene were weighed out into a 250mL three-neck flask. This suspension was subject to magnetic stirring under nitrogen atmosphere while heating to 320°C using a heating mantle and temperature controller. This solution was allowed to reflux for 2 hrs and then cooled to room temperature before purification.

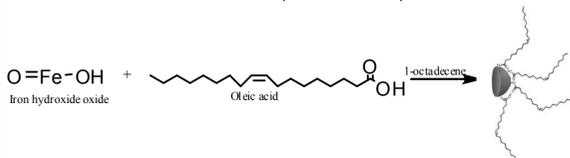


Figure 1. Reaction scheme for MION synthesis using iron oxide hydroxide as the iron source and oleic acid as organic surfactant.

Excess oleic acid and unreacted FeOOH were removed by extracting the nanoparticle suspension 3x with a 1:1 (v/v) methanol:chloroform solution. Acetone was then used to precipitate the nanoparticles from the octadecene phase. The nanoparticles were then isolated using a 0.5T magnet and redispersed in chloroform.

#### Poly (maleic anhydride-alt-1-octadecene) (PMAO)-co-poly (ethylene glycol) (PEG) (PMAO-PEG) synthesis and conjugation to MION

PMAO-PEG was synthesized by combining PMAO and methoxy PEG in a 1:30 molar ratio and refluxing in chloroform at 60°C for 18 hrs under nitrogen atmosphere using a few drops of sulfuric acid as catalyst (Figure 2). Chloroform was removed from the polymer by rotary evaporation.

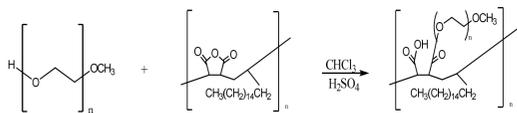


Figure 2. Reaction scheme for PMAO-PEG synthesis.

MION particles were then reacted with PMAO-PEG in chloroform for 24hrs using an approximate 1:10 molar ratio. The resulting PMAO-PEG coated nanoparticles were then made water soluble by adding an equal volume of water as there was chloroform present and slowly evaporating the chloroform by rotary evaporation. The nanoparticle suspension in water was then dialyzed against phosphate buffered saline (PBS) using 3,500 MWCO dialysis tubing to remove trace chloroform and to enhance its solubility in PBS. The particle suspension in PBS was then dried in a vacuum oven overnight at room temperature and then resuspended at 1mg/mL in PBS.

### Characterization of MION and PMAO-PEG coated MION

#### Transmission electron microscopy (TEM)

High-energy TEM was utilized to measure the average diameter of MION particles as well as to evaluate particle dispersity and morphology. MION particles in chloroform were added to acetone. This dilute suspension was sonicated briefly and deposited onto holey carbon TEM grids. An FEI Titan 80/300 field emission electron microscope (FEI Company, Hillsboro, OR) was used for TEM imaging. ImageJ™ software (NIH, Bethesda, MD) was used to identify and analyze MION particles present in TEM micrographs.

#### Atomic force microscopy (AFM)

AFM was performed on MION particles as a complementary technique to TEM to evaluate particle diameters and morphology. AFM samples were prepared by applying a suspension of MION particles in water to a freshly cleaved 3-aminopropyltrimethoxysilane (APTMS)-functionalized mica substrate, drying the substrate, and imaging it in air in tapping mode AFM using silicon OTESPA probes (Veeco Probes, Camarillo, CA) with nominal tip radius of 10nm, nominal spring constant of 42N/m, and resonance frequency of 351kHz. A BioScope II AFM (Veeco Instruments, Santa Barbara, CA) was used for this work. Nanoscope 6.13b26 software (Digital Instruments/Veeco) was used to display and analyze all AFM data.

#### Fourier transform infrared spectroscopy (FTIR)

FTIR samples of PMAO and PMAO-PEG polymers as well as MION particles were prepared by dissolving each separately in chloroform and then depositing the solutions onto the KBr aperture of Real Crystal® IR sample cards. PMAO-PEG coated MION particles were prepared similarly after dissolution in PBS. The solutions were then dried to form thin films prior to acquiring FTIR spectra using a Thermo Nicolet FTIR spectrometer.

#### Dynamic light scattering (DLS)

DLS was performed using a DynaPro99 (Wyatt Technology Corp., Santa Barbara, CA) dynamic light scattering instrument. Dilute suspensions of MION particles in chloroform and PMAO-PEG coated MION particles in PBS were briefly sonicated before analysis. The polymer coated particles were additionally filtered using a 0.22µm syringe filter. A 2s acquisition time was used and the temperature was maintained at 22°C for all samples. At least 30 measurements were collected for each sample and data was analyzed using Dynamics v5.26.60 (Wyatt Technology Corp.).

### Cell culture and Prussian blue staining

MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), cultured using RPMI-1640 medium (ATCC) adjusted to contain 10% (v/v) Fetalplex (Gemini Bioproducts, West Sacramento, CA), and incubated at 37°C with 5% CO<sub>2</sub> for 5 hrs. PMAO-PEG coated MION particles were then added to cells at final concentrations of 0.005mg/mL, 0.01mg/mL, 0.02mg/mL, 0.05mg/mL, and 0.1mg/mL. After 1hr nanoparticle exposure, the cells were stained with 2% potassium ferrocyanide in 6% HCl in PBS for 30 min in the dark. The cells were then harvested by trypsinization, fixed in 4% glutaraldehyde in PBS, and imaged using a Nikon (Nikon, Melville, NY) Optiphot inverted fluorescence microscope in brightfield mode under 20x and 40x magnification.

## Results

TEM micrographs indicated that the MION particles were monodisperse with a narrow size distribution and near spherical morphology (Figure 3).

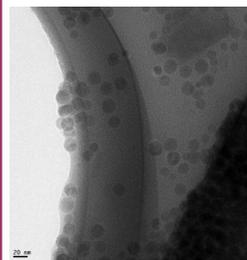


Figure 3. TEM micrograph of MION particles deposited from acetone onto a holey carbon TEM grid.

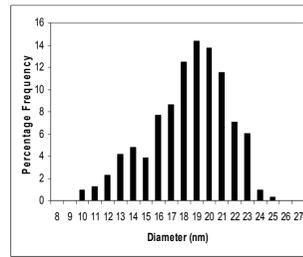


Figure 4. Particle size distribution for MION particles identified in TEM micrographs.

Analysis of 313 particles present in TEM micrographs indicate an average particle diameter of 18nm +/- 3nm (Figure 4).

AFM images of the MION particles also indicated a near spherical particle morphology (Figure 5). As seen in Figure 6, cross section analysis was performed on the MION particles identified in Figure 5. The diameters determined by this analysis are greater than those measured by TEM, likely due to convolution of the cantilever tip with the MION particles.

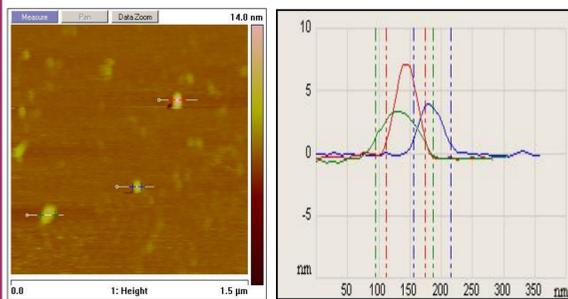


Figure 5. AFM tapping mode height image of MION particles dried onto an APTMS-functionalized mica substrate. Scan size=1.5µm, scan rate=1.15Hz, z-range=14nm.

Figure 6. Cross section analysis of selected particles identified in Figure 5. Observed particle diameters were 58nm, 64nm, and 93nm for particles bounded by blue, red, and green brackets, respectively.

Hydrodynamic radii (Rh) of MION particles, calculated by DLS via the Stokes-Einstein equation, were generally greater than those measured by TEM because Rh values are a measure of the radius of a theoretical dynamic hard sphere hydrated by water molecules and solvent ions. Uncoated MION particles dispersed in chloroform were found to have an average Rh value of 13.22nm +/- 4.5nm, corresponding to an average 26.4nm diameter +/- 4.5nm (Figure 7). PMAO-PEG coated MION particles dispersed in PBS (Rh=33.9nm +/- 3.9nm, diameter=67.8nm +/- 7.8nm) had greater Rh values as expected due to the polymer coating (Figure 8). The second particle population with Rh=150.2 indicates that some particle aggregation is occurring.

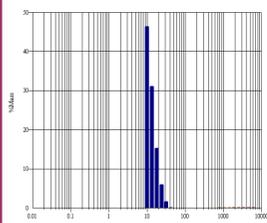


Figure 7. DLS particle size distribution for MION particles dispersed in chloroform (Rh=13.22nm +/- 4.47nm (polydispersity =33.8%) based on mass distribution).

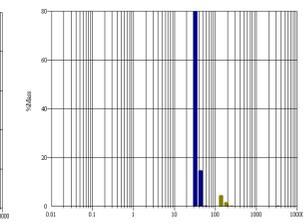


Figure 8. Figure 7. DLS particle size distribution for PMAO-PEG coated MION particles in PBS (Rh=33.9nm +/- 3.9nm, polydispersity =11.6%) based on mass distribution.

FTIR spectra obtained for PMAO and PMAO-PEG indicate that some PMAO-PEG was formed during the synthesis reaction (Figure 9). Of particular interest in the spectra are two peaks. The band at 1740 cm<sup>-1</sup>, which is expected to be greater in magnitude for PMAO-PEG than for PMAO, corresponds to free carboxyl groups that result from maleic anhydride ring opening during the reaction. The band at 1775 cm<sup>-1</sup> should become less intense during the reaction and corresponds to the disappearance of the anhydride ring. Although the disappearance of the anhydride ring is observed for the PMAO-PEG product, the resultant appearance of free carboxyl groups is not evident. However, the band at 1740 cm<sup>-1</sup> is expected to become greater in intensity once water is introduced to PMAO-PEG.

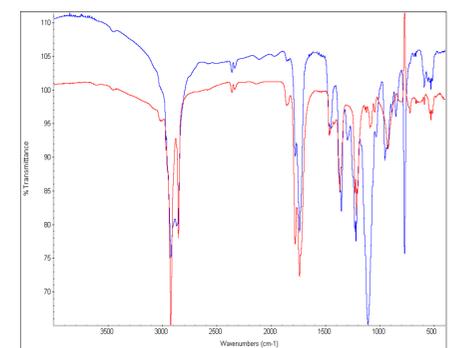


Figure 9. FTIR spectra of PMAO (red trace) and PMAO-PEG (blue trace). Band assignments: 1100= -CH<sub>2</sub>-O- of methoxy PEG, 1740=free COOH groups in PMAO-PEG resulting from PMAO anhydride ring opening, 1775=intact maleic anhydride C=O bond, 2850, 2920=C-H bonds present in CH<sub>2</sub> groups of both polymers.

Prussian blue staining of MDA-MB-231 breast cancer cells exposed to various concentrations (5-100µg/mL) PMAO-PEG coated MION particles was done to determine the particle concentration that qualitatively showed particle uptake in cells. Prussian Blue staining is intended to result in a blue coloration of any iron-containing compounds in cell populations, including MION particles and endogenous iron. With the first experiment, it was difficult to ascertain by this method whether any MION particles were taken up by the cells (data not shown). The cells were, however, 99% viable after 1hr PMAO-PEG MION exposure. Previous studies showed very intense coloration of the cells after uptake of non-coated MION (Figure 10).

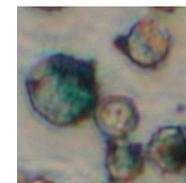


Figure 10. Brightfield microscopy image of Prussian blue stained cells after 2hr exposure to non-coated MION particles.

## Conclusions

Synthesized monodisperse iron oxide nanoparticles had a narrow size distribution due to the transfer from octadecene to PBS solvent by coating with the amphiphilic copolymer PMAO-PEG. The PMAO unit has a hydrophobic octadecene side chain for interacting with the oleic acid surfactant on the MION surface and provides for a free carboxyl functional group for further conjugation to biomolecules including peptides and nucleic acids. The PEG unit enhances the solubility of these MION in aqueous solutions. Preliminary studies with Prussian Blue staining show that even uncoated MION can be taken up successfully by cells, while PEG-coated MION appear to be non-cytotoxic (99% viability reported). These nanoparticles have the potential for use in biological systems although long-term cytotoxicity studies are needed.

## Future Work

### Peptide conjugation for cell targeting/enhanced particle uptake in specific cell types

Peptide conjugation to PMAO-PEG coated MION particles is planned to target the nanoparticle constructs to specific breast cancer cell types for more efficient nanoparticle dosing to cells. Peptides with free amino groups can be directly conjugated to the free carboxyl groups present in PMAO-PEG that were created by maleic anhydride ring opening. Alternately, the free PMAO-PEG carboxyl groups could be first conjugated to a diamine linker and then a peptide containing free carboxyl groups could be conjugated to the nanoparticles via this linker molecule.

### DNA barcoding of PMAO-PEG MION for *in vitro* and *in vivo* particle detection

Because iron oxide nanoparticles are difficult to detect *in vitro* owing to their extreme small size, the 26-mer oligonucleotide:

5'-GTGAAGCGTCAAACGACAATCGAACG-3'

(non-endogenous to the mouse or human genomes) will be conjugated to PMAO-PEG coated MION particles. Its unique nucleotide sequence will allow the nanoparticle constructs to be recovered from tissue samples and detected by PCR amplification and subsequent DNA sequencing of the oligonucleotide "bar codes". This methodology is expected to be more definitive for detecting nanoparticles *in-vitro* than Prussian blue staining.

### AFM imaging of PMAO-PEG MION internalization

Real-time nanoparticle trafficking into breast cancer cells will be monitored by performing AFM on cells incubated in the presence of PMAO-PEG MION particles for various time periods. Because it may be difficult to discern nanoparticles on cell surfaces by traditional AFM, magnetic force microscopy (MFM) will be used for this work. MFM utilizes a cantilever with a magnetic tip that interacts with magnetic materials.

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