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Biodistribution and imaging of fluorescently-tagged iron oxide nanoparticles in a breast cancer mouse model

Jennifer A. Tate^a, Mark D. Savellano^b, and P. Jack Hoopes^b

^aThayer School of Engineering, 14 Engineering Drive, Hanover, NH, USA 03755-8000

^bDartmouth Medical School, 1 Rope Ferry Road, Hanover, NH USA 03755-1404

Abstract

Iron oxide nanoparticle (IONP) hyperthermia is an emerging treatment that shows great potential as a cancer therapy both alone and in synergy with conventional modalities. Pre-clinical studies are attempting to elucidate the mechanisms of action and distributions of IONP in various *in vitro* and *in vivo* models, however these studies would greatly benefit from real-time imaging of IONP locations both in cellular and in mammalian systems. To this end, fluorescently-tagged IONP (fIONP) have been employed for real time tracking and co-registration of IONP with iron content. Starch-coated IONP were fluorescently-tagged, purified and analyzed for fluorescent signal at various concentrations. fIONP were incubated with MTGB cells for varying times and cellular uptake analyzed using confocal microscopy, flow cytometry and inductively-coupled plasma mass spectrometry (ICP-MS). fIONP were also injected into a bilateral mouse tumor model for radiation modification of tumor tissue and enhanced fIONP deposition assessed using a Xenogen IVIS fluorescent imager. Results demonstrated that fIONP concentrations *in vitro* correlated with ICPMS iron readings. fIONP could be tracked *in vitro* as well as in tissue samples from an *in vivo* model. Future work will employ whole animal fluorescent imaging to track the biodistribution of fIONP over time.

Keywords

iron oxide; magnetite; magnetic; nanoparticle; hyperthermia; biodistribution; *in vivo*; fluorescence

1. INTRODUCTION

Iron oxide nanoparticle hyperthermia has shown promise in recent years as an alternative to conventional hyperthermia therapy for cancer treatment. IONP hyperthermia utilizes iron oxide-core nanoparticles that, when activated by an external alternating magnetic field (AMF), give off energy in the form of heat (1). Due to the IONPs' cellular size-scale and their ability to distribute a heat dose over a wide range of geometries, IONP hyperthermia has the potential for enhanced specificity of heat dose as well as better-defined tumor margins compared to conventional hyperthermia delivery methods. At sub-lethal doses,

IONP hyperthermia may also enhance conventional radiation and chemotherapy by delivering synergistic energy on the cellular level (2-4).

Clinical treatment planning for IONP hyperthermia therapy, whether through directly-injected IONP or systemically-administered IONP, must rely on tissue and intratumoral distributions of IONP in order to accurately predict dosages with AMF activation. IONP imaging using magnetic resonance or x-ray computed tomography is currently being pursued to this end, however these standard imaging modalities require further optimization in order to detect ferromagnetic-core IONP at high and low doses with adequate resolution. Fluorescence, a powerful pre-clinical imaging modality, provides a much more accessible alternative up front as compared to clinical imaging systems.

In the following pilot studies, fluorescent IONP (fIONP) are tested in a variety of situations, from *in vitro* using flow cytometry and confocal microscopy to *in vivo* with a small animal imaging system. Some of the challenges related to fluorescent imaging of fIONP are discussed along with the presentation of preliminary results.

2. METHODOLOGY

2.1 Mice used for study

All mice are cared for according to approved IACUC animal protocol. Female nude or C3H mice were obtained from the Jackson Laboratories (Bar Harbor, Maine). At 8-11 weeks old, mice were implanted in the subcutaneous flank with 1 million MTGB mouse mammary carcinoma cells in 100 μ l serum-free alpha-MEM using a 1ml syringe and a 25G needle. Mice were monitored every three days until the tumor reaches 50mm³, upon which mice were measured once every two days. Mice were put on study once the tumor volume reaches between 100-200mm³ as measured by calipers and calculated using an ellipsoid approximation.

2.2 Particles used for the studies

All nanoparticles used for these studies were purchased from Micromod Partikeltechnologie GmbH (18119 Rostock-Warnemuende, GERMANY). Fluorescently-tagged BNF-starch IONP were obtained and characterized using ferrozine assay, ICP-MS and the Malvern Zetasizer, with fluorescence measured using a spectrophotometer and absorbance assessed using a NanoDrop. Stock iron concentrations vary from batch to batch and were validated in house before utilizing a given batch. Batches were stored at 4°C and used within the reported shelf life.

2.3 In Vitro fIONP administration

MTGB cells were maintained in complete alpha-MEM media containing fetal bovine serum. Cells were grown on either flasks, cell adherence-treated confocal coverslips or on coverslip-bottom plates. At the appropriate timepoints, cell media was removed and replaced with media containing fluorescent-conjugated IONP. After fixed incubation times, cells were either imaged live on a confocal microscope, or coverslips were washed, removed, fixed and mounted using DAPI-containing ProLong® Gold Antifade reagent (Invitrogen).

2.4 In Vivo fIONP administration

Once a mouse reached treatment size, fIONP dose was calculated using mouse body mass (g). All other iron concentrations were normalized to the lowest concentration to ensure equal volume scaling. fIONP stocks were made isotonic with the addition of salts (PBS, NaCl) prior to *in vivo* use. A 1ml syringe with 30g needle was loaded with the full injection dose and half the volume marked off. Mice were anesthetized using either isoflurane or a mixture of ketamine and xylazine. The appropriate fIONP dose was then injected intravenously into the tail vein, followed by imaging.

2.5 Tissue harvesting

At sacrifice, mice were anesthetized using an overdose of isoflurane. At least 10 seconds after respiration is no longer observed, the mouse was checked for pain stimulus response by pinching the leg. After ascertaining lack of vital responses, mice were dissected and slices and organs imaged to assess fluorescent IONP content.

2.6 Inductively Coupled Plasma Mass Spectrometry (ICP-MS) digestion protocol

The following digestion procedure occurs in a fume hood due to ammonia production. A 1:3 volumetric mixture of stock concentration trace metal grades HCl to nitric acid (Fisher Scientific) was prepared in a 50ml conical tube. The acid mixture was immediately added to the post-weighed conical tubes containing harvested tissues. For cell-only harvests, samples were digested in stock trace metal grade HCl only. A DORM-3 standard, tumor, spleen, heart and lung tissues received at least 2ml of the acid mixture while blood and kidney tissues received at least 5ml and livers receive at least 7ml. The tissues were allowed to digest at room temperature, caps vented, for at least one hour before being placed on a heat block and incubated at 60°C for at least two hours. Vials were sealed and inverted at least twice during the heating process. Following heating, samples were allowed to cool a minimum of overnight before caps were sealed and all vials weighed and numbered. Sample data was logged in a spreadsheet and samples are submitted to the Dartmouth Trace Elements core for iron content analysis.

3. RESULTS

In vitro characterization of fIONP showed both a peak excitation absorbance shift (example Figure 1 for Dylight 633 from 638nm to 620nm) upon fluorophore conjugation as well as significant background signal at wavelengths below the near-infrared range with increasing concentrations. Scattering interference from IONP crystal cores as well as the peak shift in dye wavelength making it difficult to directly quantify dye in this wavelength range on the IONP surface; an indirect quantification using remaining unbound dye following fluorophore conjugation would avoid this interference. Using dyes with an excitation and emission peak above 700, though some interference is unavoidable at high concentrations.

The core scattering effect can also be observed in Figure 2a, co-registering well with detected fluorescent signal of fIONP aggregates in Figure 2b. The effect of core scattering on IONP fluorescence in these aggregates is not known, however care must be taken to use an orthogonal method of quantification such as iron analysis in order to verify IONP

concentrations for various intracellular particle and aggregate configurations. Figure 2c demonstrates signal “quenching” on an *in vivo* imaging system, where at the highest measured concentration there is a marked decrease in fluorescent signal assumedly due to interference from the IONP crystal core.

Fluorescent signal correlates with intracellular iron content, as is evident in Figure 3 where highly-fluorescent MTGB cells following incubation with fIONP are shown to also have the highest intracellular iron.

Using confocal microscopy, incubation with fIONP revealed increasing intracellular fluorescence with increased incubation times. At 30 minutes (Figure 4a), cells display minimal fluorescent signal in the cytoplasm. By 4 hours post-incubation (Figure 4b), cells show markedly more intracellular fluorescence with bright punctuate groupings indicative of nanoparticle clusters or aggregates as well as larger collections with a more diffuse signal that may be nanoparticle-loaded vesicles or collections in adjacent slices. At 24 hours (Figure 4 c), cells displayed markedly brighter cytoplasmic regions, either correlating to an abundance of diffuse single particles or free dye resulting from the intra-vesicular breakdown of fIONP coatings. Punctate aggregates are still visible and bright; there appear to be many more intracellular clusters.

fIONP were additionally used in follow-up to a study comparing intratumoral IONP deposition in irradiated and non-irradiated mice (2). In the study, female C3H mice bearing bilateral flank MTGB tumors had the right flank tumor irradiated with a dose of 15Gy with the left tumor serving as a control. Three days following irradiation, fIONP were injected intravenously via tail vein. Tumors were excised and imaged 24 hours later to assess fIONP content. Figure 5 shows the excised flank tumor pairs, with control tumor on the left and irradiated tumor on the right. An example control mouse is included in the image where neither tumor received radiation. All irradiated tumors displayed increased fluorescence over their non-irradiated counterparts at equal fIONP dose, suggesting that fIONP accumulation following sub-lethal radiation modification of the tumor environment is significantly enhanced.

4. DISCUSSION

Initial studies characterizing fIONP found that choosing the appropriate fluorophore wavelengths was crucial to accurate quantification. Core scattering from the iron oxide crystals interfered with lower wavelength fluorescent signal, however higher wavelength fluorophores such as near-IR dyes could largely avoid this effect. Though lower-wavelength fluorophores such as FITC or TRITC are accessible for many *in vitro* experiments, near-IR dyes are favorable for *in vivo* imaging purposes. Additional considerations for the quantification of fIONP fluorescent signal include peak shifts following conjugation, requiring adjusted standard curves, as well as signal degradation over time in a living system setting. Fixed samples of cells containing fIONP were easily visualized on a confocal microscope, however, suggesting good conjugate stability.

In vitro, fluorescent signal correlated well with cellular iron content (Figure 3). Similar results were observed *in vivo* (not shown), though incurring high variability in background fluorescence due to tissue autofluorescence and low fIONP dose. The relatively high fluorescent signal to iron content implies that higher doses of fIONP should provide even better contrast to noise ratio. Even with this, a pre-injection image is useful to determine background concentrations, especially in the abdominal region where even specially-formulated rodent chow for fluorescent imaging provides some background signal. Accurate quantification of fIONP in abdominally-located organs such as the liver and spleen is crucial to assessing fIONP biodistribution, as IONP have been shown to accumulate largely in the reticuloendothelial system (5, 6). The overall background will necessarily change over time with tissue scattering and as nanoparticle and fluorophore degrade. It may be possible to track the disassociation of fIONP coating from iron core by monitoring the respective fluorescent signal and iron concentrations, though challenges exist in quantifying this observation since fluorescent signal will degrade over time.

More sophisticated 3D small animal imaging systems, some incorporating CT co-registration, have the potential for real time biodistribution and quantification of fIONP in specific organ compartments. These systems are expensive, however, and standard 2D imaging techniques require further optimization in order to quantify fIONP concentrations with acceptable or comparable accuracy to ICP-MS and histology.

5. CONCLUSIONS AND FUTURE DIRECTIONS

In summary, fIONP provide an alternative economical method for tracking nanoparticle distribution and uptake in *in vitro* and *in vivo* models. fIONP fluorescence has been shown to function as a quantitative metric for IONP content *in vitro* in both live and fixed cell culture scenarios for fluorescent microscopy and flow cytometry. *In vivo*, fIONP show similar quantitative metric capabilities in excised tissue as well as histologically, however real-time imaging is still subject to its inherent limitations albeit benefiting from a high degree of fluorescent labeling.

Though fluorescent tagging can be applied to a variety of functionalized IONP, the degree of labeling and the subsequent change in surface characteristics should be taken into account. Highly fluorescently-labeling IONP can alter the hydrodynamic size as well as zeta potential of a given particle type, rendering it non-comparable to its clinically-relevant base particle. Fluorescent labeling could also be enacted on targeted and/or PEG-ylated particles, but the location of the fluorescent label (eg. on the particle surface, on the terminal end of the PEG chain, or attached to the targeting moiety) must be additionally considered. Despite these concerns, fluorescently-tagged IONP have proven a useful pre-clinical method for imaging and monitoring of nanoparticles. The ease of modification and accessibility of fluorescent technology in the laboratory make fIONP technology worth pursuing in the path to clinical translation.

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Fig 1.

NanoDrop measurements of fIONP absorbance at different standard dilutions, the highest in green and lowest in black. Conjugated Dylight 633 dye displays a peak absorbance at 620nm compared to the reported peak at 638nm. At high concentrations, the IONP core shows significant absorption, which affects the standard curve for conjugated dye concentration.

Figure 2.

a) Backscatter image TRITC-conjugated fIONP using a Zeiss AxioplanII confocal microscope, b) Fluorescence image of TRITC-conjugated fIONP using a Zeiss AxioplanII confocal microscope, and c) CCD camera image of TRITC-conjugated fIONP 1:2 serial dilutions from 5 mg fIONP/ml (right) to buffer only (left).

Figure 3.

Top: MTGB cells were incubated TRITC-conjugate fionp for 4 or 24 hours, harvested and then sorted into three gated along with control, fionp-free cells using a FACS Aria cell sorter. Sorted cell were then analyzed for iron content using IC₂-MS-analyzed iron content showd that cells with the highest fluorescent signal contained the most iron.

Figure 4.

Composite images of MTGB cells grown on confocal coverslips incubated with fIONP and harvested after a) 30 minutes, b) 4 hours and c) 24 hours of incubation. At harvest, coverslips were washed, fixed and mounted using DAPI-containing ProLong® Gold Antifade reagent. The 24 hour timepoint displayed the highest degree of DAPI staining despite all samples being fixed and stored in mount medium for 24 hours. Slices are 1µm thick.

Figure 5.

C3H mice were given bilateral flank MTGB tumor implants. Upon the second tumor reaching treatment size, mice 1, 2 and 4 had their right flank tumors irradiated using a linear accelerator at a dose of 15Gy; mouse 3 had neither tumor irradiated. Three days post-irradiation, all mice were given intravenous fIONP. 24 hours post-injection, tumor pairs were harvested and imaged on a Xenogen IVIS system. Increased fluorescent signal was visible in irradiated tumor as compared to the control tumor 24 hours after IV administration.