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Magnetic nanoparticle biodistribution following intratumoral administration

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Abstract

Recently, heat generated by iron oxide nanoparticles (IONP) stimulated by an alternating magnetic field (AMF) has shown promise in the treatment of cancer. To determine the mechanism of nanoparticle-induced cytotoxicity, the physical association of the cancer cells and the nanoparticles must be determined. We have used transmission electron microscopy (TEM) to define the time dependent cellular uptake of intratumorally administered dextran-coated, core-shell configuration IONP having a mean hydrodynamic diameter of 100-130 nm in a murine breast adenocarcinoma cell line (MTG-B) in vivo. Tumors averaging volumes of 115 mm³ were injected with iron oxide nanoparticles. The tumors were then excised and fixed for TEM at time 0.1 to 120 hours post IONP injection. Intracellular uptake of IONP was 5.0, 48.8 and 91.1% uptake at one, two and four hours post-injection of IONP, respectively. This information is essential for the effective use of IONP hyperthermia in cancer treatment.

Keywords

magnetic nanoparticle; tumor; TEM; biodistribution; breast adenocarcinoma

1. Background

It is well known that exposing tumor cells to modestly elevated temperatures sensitizes them to chemotherapy and radiation and, depending on temperature and exposure time, decreases their viability [1] [2]. The first use of magnetic hyperthermia on tumors was reported in 1957, when Gilchrist et al. demonstrated through in vitro experiments that 5 mg of 20-100 nm diameter Fe₂O₃ nanoparticles in lymph nodes (47 mg of Fe₂O₃ per gram of tissue) could produce a temperature rise of 14°C in an AMF of 200-240 Oe at 1.2 MHz [3]. Recent advances in nanoparticle technology have allowed for a promising cell-targeted form of therapeutic hyperthermia [4] [5] [6] [7].

When appropriately coated, IONP demonstrate excellent biocompatibility with limited toxicity [8]. At the same time, these nanoparticles can be constructed to have a very high specific absorption rate (SAR), resulting in excellent heating properties and targeting capabilities [9]. Considerable work has been done to elucidate the biologic, therapeutic and materials properties of matrix-configuration iron oxide nanoparticles [10] [11] [12]. This

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configuration of nanoparticle is typically composed of several, small (3-15 nm) iron oxide crystals distributed within a polymer matrix [8].

Another configuration of iron oxide nanoparticle, most commonly used in AMF-induced hyperthermia, is the core-shell configuration. In this configuration, larger magnetic iron oxide cores (up to approximately 50 nm) are coated with a polymer shell [9]. This configuration typically demonstrates greater SAR than the matrix-configuration IONP [9] [13] [14] and, thus, are well-suited to therapeutic hyperthermia applications. To date, few, if any, studies have demonstrated the biodistribution of core-shell IONP [15]. Thus, additional work is necessary to demonstrate the interaction of core-shell IONP with cells and tissues. In this study, we report on the cellular uptake parameters of dextran-coated, iron oxide core-shell nanoparticles.

Theoretical models have been published which have shown that currently available nanoparticles of the concentrations used for nanoparticle hyperthermia will be unable to generate sufficient heating to achieve global hyperthermia [16]. These models assume homogeneous distributions of nanoparticles within a sample; our study suggests that this model is not directly applicable to many biological systems. In addition, inter-particle distance has been shown to be a critical parameter for the generation of heat [17]; this study, and others, have shown that IONP localize in vacuoles within cells. In addition, studies have demonstrated that iron oxide nanoparticles can be used to achieve global hyperthermia within tissues [6] [17]. Our data suggest that the distribution of nanoparticles within tumors is not homogeneous. Rather, the nanoparticles aggregate and this aggregation may aid in local heat deposition.

2. Materials and Methods

2.1 Nanoparticles

The nanoparticles utilized in this experiment were dextran-coated, iron oxide (magnetite, Fe_3O_4)-core BNF® nanoparticles with an average hydrodynamic diameter range of 100-130 nm (MicroMod GmbH, Rostock, Germany). The mean magnetite core diameter was approximately 45 nm. The nanoparticle iron concentration was 14.5 mg Fe/ml (33 mg nanoparticle/ml) in deionized water. The synthesis of these nanoparticles was described by Gruettner, et al [9] and a complete description of their physical characteristics has been published [17].

2.2 Cells

A murine breast adenocarcinoma cell line (MTG-B) [18] was cultured in the Alpha modification of Eagle's Minimal Essential Medium (MEM, HyClone Laboratories, Inc.) with 1% penicillin/streptomycin (Pen-Strep, HyClone Laboratories, Inc., Logan, UT, USA), 1% L-glutamine (Mediatech, Inc., Manassas, VA), and 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc.) at 37 degrees Celsius in 5% CO_2 atmosphere in an incubator (Queue Systems Inc., Parkersburg, VA, USA).

2.3 Animal Tumor Model

This experiment was approved by Dartmouth's Institutional Animal Care and Use Committee and all animals were treated humanely, in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). To prepare cells for implantation in mice, the cells were exposed to trypsin (0.25% trypsin in EDTA, HyClone Laboratory, Inc.), stained with trypan blue (Hyclone Laboratories, Inc.), counted using a hemocytometer (Fisher Scientific Inc. Pittsburgh, PA, USA), and then re-suspended in (serum-free, L-glutamine-free, Pen-Strep-free) Alpha MEM

media at a concentration of 10^7 cells/mL. For each tumor, 100 μ L of this solution was injected intradermally into the shaved flanks (one or two tumors per mouse) of female C3H/HE mice (The Jackson Laboratory, Bar Harbor, ME, USA), as described in table 1. Three orthogonal tumor measurements were taken with a digital caliper every two days and the tumor volume was determined using the equation for the volume of an ellipsoid. When tumors reached volumes of greater than 50 mm³ they were considered of appropriate size for analysis.

Nanoparticles (5 mg Fe/cm³ tumor) were injected using a 30-gauge needle (0.34 μ L of nanoparticle solution per mm³ of tumor). The tip of the needle was advanced into the center of the tumor and the nanoparticle suspension injected over the course of 30 seconds. The tip of the needle remained in place for five minutes post-injection to optimize nanoparticle distribution. Animals were euthanized and the tumors were excised, sectioned, fixed overnight, and processed for TEM at the pre-determined post-inject time endpoint (table 2).

2.4 Transmission Electron Microscopy

Tumor tissue samples were fixed in 200 μ L of 4% glutaraldehyde (Ted Pella, Inc., Redding, CA, USA) solution overnight and transferred to 200 μ L of 0.1 M sodium cacodylate buffer solution (pH 7.4, Ted Pella, Inc.) after three wash cycles with buffer. The samples were prepared for TEM (FEI Company Tecnai F20 FEG TEM operating at 100 kV) at the Dartmouth College Electron Microscopy Facility. Either L.R. White (Polysciences, Inc., Warrington, PA, USA) or Poly/Bed-812 (Polysciences, Inc.) was used as an embedding resin. Samples were stained with 4% osmium tetroxide (Ted Pella, Inc.) and en-bloc stained with 2% uranyl acetate (Ted Pella, Inc.) for one hour, each. Thin sections of 100-110 nm from each tumor sample were cut using a Leica Ultra-Cut Microtome (Leica Microsystems GmbH, Wetzlar, Germany).

2.5 Image Analysis to Quantify Uptake

To quantify nanoparticle uptake, computer code was written using Matlab 7.6.0.324 (The Mathworks, Inc., Natick, MA, USA) Image Processing Toolbox. Ten low-magnification (5000x), randomly-chosen TEM fields from each tumor sample were digitally photographed. This magnification allowed for the assessment of multiple cells and associated extracellular spaces within one field of view. Due to electron density similarities—but morphologic differences—of nuclear chromatin and IONP, nuclei were manually excluded from images using a digital computer input tablet (Wacom Technology Corporation, Vancouver, WA, USA). Nanoparticles were never present within nuclei, so this exclusion did not affect quantification accuracy. Artifacts from sample preparation were also excluded. The images of the cells were then manually segmented along their plasma membranes in order to separately quantify internal and external IONP.

Due to the extreme electron density of the IONP, we were able to segment each image to identify only IONP in a binary map of the intracellular region of interest (ROI). In the binary ROI, the nanoparticles were given a value of 0 and all other material a value of 1. This ROI map was inverted and summed to count the number of pixels corresponding to intracellular IONP. In a similar fashion, the extracellular space was analyzed using the same grayscale threshold value as the intracellular IONP. Once the pixels corresponding to nanoparticles were determined, an overlay was created to highlight the pixels determined to be nanoparticles and manual confirmation was completed. Using this analysis technique, the ratio of internal vs. external nanoparticles was quantified.

For each tumor sample (three tumors per time point) at times one, two, three, four, five and six hours, the total number of pixels corresponding to intracellular and extracellular

nanoparticles was independently quantified. The percentage of intracellular vs. extracellular IONP was then determined for each sample.

3. Results

Within the first hour post-injection, 95% of the observed IONP are either associated with the external plasma membrane or within the extracellular space (figure 1). The first indication of significant particle uptake by cells occurs two hours after injection when 48.8 ± 14 % of the nanoparticles are observed within the cells.

Four hours after IONP injection into the tumor, virtually all (91.1 ± 9.6 %) observed nanoparticles are present within tumor cells. The nanoparticle aggregates have become more ordered due to nanoparticle segregation within intra-cytoplasmic vesicles. Figure 2 demonstrates the intermediate time point of three hours. After six hours of incubation, virtually no IONP are observed outside the cells (figure 3). As seen at one day after injection of nanoparticles into the tumors, the nanoparticles are seemingly exclusively contained within intracellular intra-cytoplasmic vesicles (figure 3). The quantification of IONP position for time points one through six hours is presented in figure 4.

4. Discussion

These data suggest that virtually all IONP uptake occurs between one and four hours after intratumoral injection. These data suggest that there are two time domains for intratumorally delivered IONP hyperthermia. Dennis et al. demonstrated that aggregated IONP result in improved heating characteristics when compared with non-aggregated IONP [14]. Thus, once tumor cells have aggregated IONP intracellularly, more heat will be deposited into the tumor upon AMF activation with the same field strength and frequency, resulting in greater tumor cytotoxicity.

Multiple TEM sections of each tumor sample are examined. The dimension of the individual TEM sections is 5 mm $\times$ 5 mm, at a thickness of 100 nm. While the total volume of tumors sampled with each TEM is relatively small and is assessed in a two dimensional manner, the use of multiple sections over a small region allows for a reasonably accurate 3-D assessment of an individual sample.

These data are the first to describe the temporal and spatial relationship of intratumorally delivered IONP. We demonstrate that nanoparticles are, in fact, heterogeneously distributed on a cellular level within intra-cytoplasmic vesicles. Based on the observations made in this study, there does not appear to be a specific anatomic location of the nanoparticles within cells. It is well known that inter-particle interactions affect the magnetic properties of nanoparticles, which results in aggregated IONP displaying higher heating rates [14]. These improved heating effects may lead to increased cytotoxicity and tumor control and are the focus of current studies.

5. Conclusions

We have demonstrated that core-shell magnetic iron oxide nanoparticles of average hydrodynamic diameter of approximately 100 nm and coated with dextran and rapidly internalized (90% within four hours) by tumors cells *in vivo* (figure 5). Once taken up by tumor cells, the nanoparticles are trafficked together into large collections. These results will inform future work using magnetic nanoparticles activated with alternating magnetic fields to treat tumors.

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Figure 1. Transmission Electron Micrographs of In vivo murine mammary adenocarcinoma cells. **a:** Tumor cells without IONPs. **b:** Tumor cells five minutes following intra-tumor administration of IONP. The vast majority of IONP are aggregated in the extracellular space (arrow). **c:** Tumor cells one hour following intra-tumor administration of the nanoparticles. Larger interstitial aggregates of nanoparticles can be seen (arrow).

Figure 2.

Three hours following intra-tumor injection of IONP into a mouse mammary adenocarcinoma, a large number of IONP are aggregated within (black arrow) and outside (white arrow) tumor cells. **a:** Although some IONP remain outside of the cells, most IONPs are located within large intra-cytoplasmic vesicles. **b:** This micrograph demonstrates plasma membrane associated IONP and IONP in the process of trafficking through the membrane and cytoplasmic aggregation.

Figure 3.

TEM of a mouse mammary adenocarcinoma cell demonstrating aggregated IONPs within the cytoplasm (black arrow) and adjacent to the nuclear envelope (white arrow), 24 hours after administration. All IONPs were either aggregated inside cells or had been eliminated from the tumor extracellular space.

Figure 4.

One hour post intratumoral IONP injection (5 mg Fe/cm^3 tumor), approximately 5% of nanoparticles were found to be intracellular (TEM assessment), whereas 4 hours post injection approximately 90% are intracellular. There is no additional uptake at 6 hours post injection. Error bars indicate standard deviation of the mean.

Figure 5.

These figures demonstrate IONP movement from the extracellular location (1, left figure) to semi-aggregated intracellular (1) and extracellular (2) locations (center figure) to entirely intracellular aggregation (2, right figure). The intratumoral post-injection time is indicated beneath the photographs.

Table 1

This table shows the characteristics of each tumor used in the study.

Mouse	Tumor Location	Tumor Volume (mm ³)	IONP Target Injection Volume (μL)	IONP Incubation Time
AA3	Right Flank	54	N/A	Control
AA4	Left Flank	133	46	5 Minutes
AA2	Right Flank	104	36	30 Minutes
AA2	Left Flank	207	71	1 Hour
AAD3	Right Flank	70	24	1 Hour
AAD4	Right Flank	97	33	1 Hour
EMA3	Right Flank	94	32	2 Hours
EMA3	Left Flank	105	36	2 Hours
EMB1	Left Flank	107	37	2 Hours
EMA2	Left Flank	107	37	3 Hours
EMA4	Left Flank	89	31	3 Hours
EMB2	Right Flank	84	29	3 Hours
EMB1	Right Flank	101	35	4 Hours
GG4	Right Flank	208	72	4 Hours
AAE2	Left Flank	106	37	4 Hours
AAD2	Right Flank	112	39	5 Hours
AAE1	Right Flank	127	44	5 Hours
AAE3	Right Flank	145	50	5 Hours
AAE2	Right Flank	113	39	6 Hours
AAC2	Right Flank	80	28	6 Hours
AAC3	Right Flank	99	34	6 Hours
FF1	Right Flank	127	44	9 Hours
FF3	Right Flank	146	50	13 Hours
HH1	Right Flank	141	49	18 Hours
AB2	Left Flank	105	36	24 Hours
FF4	Right Flank	121	42	5 Days

Table 2

The number of tumors examined at each nanoparticle incubation time point.

Incubation Times	Number of Tumors Examined	% of Nanoparticles Intracellular ($\pm$ Standard Deviation)
Control (no IONP)	1	N/A
Five Minutes	1	0
Thirty Minutes	1	0
One Hour	3	5.0 ($\pm$ 4.6)
Two Hours	3	48.8 ($\pm$ 13.6)
Three Hours	3	70.7 ($\pm$ 13.5)
Four Hours	3	91.1 ($\pm$ 9.6)
Five Hours	3	87.7 ($\pm$ 9.5)
Six Hours	3	92.9 ($\pm$ 6.7)
Nine Hours	1	100
Thirteen Hours	1	100
Eighteen Hours	1	100
One Day	1	100
Five Days	1	100