DELETERIOUS SYNERGISTIC EFFECTS OF CONCURRENT MAGNETIC FIELD AND SUPERPARAMAGNETIC (Fe₃O₄) NANOPARTICLE EXPOSURES ON CHO-K1 CELL LINE

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While many investigations have been performed to establish a better understanding of the effects that magnetic fields and nanoparticles have on cells, the fundamental mechanisms behind the interactions are still yet unknown, and investigations on concurrent exposure are quite limited in scope. This study was therefore established to investigate the biological impact of concurrent exposure to magnetic nanoparticles and extremely-low frequency magnetic fields using an in-vitro CHO-K1 cell line model, in an easily reproducible manner to establish grounds for further in-depth mechanistic, proteomic, and genomic studies. Cells were cultured and exposed to 10nm Fe$_3$O$_4$ nanoparticles, and DC or low frequency (0Hz, 50Hz, and 100Hz) 2.0mT magnetic fields produced by a Helmholtz coil pair. The cells were then observed under confocal fluorescence microscopy, and subject to MTT biological assay to determine the synergistic effects of these concurrent exposures. No effects were observed on cell morphology or microtubule network; however, cell viability was observed to decrease more drastically under the combined effects of magnetic field and nanoparticle exposures, as compared to independent exposures alone. It was concluded that no significant difference was observed between the types of magnetic fields, and their effects on the nanoparticle exposed cells, but quite clearly there are deleterious synergistic effects of these concurrent magnetic field and nanoparticle exposure conditions.
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CHAPTER 1

INTRODUCTION

Despite numerous investigations since the 1970s, the effect of low frequency magnetic fields on cells has remained unclear and the subject of much controversy, especially because many investigators report irreproducible or even contradictory findings [1–4]. Difficulties arise in that experiments can be very difficult to repeat as many exposure systems are custom made for their respective experiments, and even minor variations in the design can induce moderate to large changes in the magnetic fields produced. Additionally, the effects of magnetic field exposures on cells are not necessarily ubiquitous, but rather may be dependent on cell line [5], cell stage, or even age of cells or their respective donors [6,7]. Induced cellular level effects may be also very small and difficult to quantify, and very minute variations in magnetic field parameters such as field intensity, direction, duration of exposure, and field frequency may have significant impacts on cell behavior [8–10]. Finally, the possible biophysical mechanisms behind any induced cellular effects are still not well known, however many theoretical models have been proposed [11].

Many studies have been presented on the effects of magnetic field exposures on various cell lines, particularly at standard distribution frequencies of 50Hz and 60Hz [12–21] and DC magnetic fields [22–25]. Additionally extensive investigations have been carried out on the toxicological effects of nanoparticle interactions with cells, and what nanoparticle properties impact those interactions [26–33]. Concurrent nanoparticle and magnetic field interactions have indeed been investigated [12,23,24,34–38], however there are no consistent and agreeable results from these studies showing specific effects of concurrent low frequency
magnetic fields, and magnetic nanoparticle exposure on cells. This study was therefore designed to investigate the biological impact of concurrent DC, or low frequency magnetic fields and magnetic nanoparticle exposures on the CHO-K1 cell line, in an easily reproducible manner, to establish grounds for further in-depth mechanistic, proteomic, and genomic studies. For the purpose of developing future studies, and for independent verification, the magnetic field exposure was conducted using commercially available components, while nanoparticle exposure procedures emulate that of well-established, previously performed toxicological studies [32,39,40]. The exposure systems, protocols, and measurements are further detailed in chapter 3. For the purpose of this experiment, the term “magnetic field” and “magnetic field intensity” refer specifically to the magnitude of magnetic flux density of the induced magnetic fields.

1.1 Objectives

The main objective of this research was to investigate the biological impact of concurrent exposure to magnetic nanoparticles (NP) and extreme-low frequency magnetic fields (ELF-MF) using an in-vitro CHO-K1 cell model. This objective was achieved through the following specific aims:

Specific aims:

1. Establish in-vitro NP, ELF-MF, and concurrent NP+MF exposure system for the CHO-K1 cell line model. Specifically DC, 50Hz, and 100Hz 2.0mT magnetic fields and 10nm spherical superparamagnetic Fe₃O₄ nanoparticles were used in this study.
2. Investigate biological impacts and perform comparative analysis of concurrent exposures against independent exposures, focusing specifically on cell viability and recovery through mitochondrial activity measurement following NP and MF treatment.

3. Investigate cell morphology and nanoparticle uptake changes in CHO-K1 cells via confocal microscopy using mEmerald-tubulin expressing CHO cells, and Rhodamine-B labeled nanoparticles.

1.2 Organization of Thesis

Chapter 2 presents information obtained through literature review of relevant scientific investigations on cellular effects of nanoparticle, magnetic field, and concurrent exposures. The first section of this chapter is dedicated specifically to current investigative efforts on cytotoxicity and nanoparticle-cell interactions, while the second section is dedicated specifically to magnetic field-cell interactions. As current investigations are limited with regard to concurrent exposures, a brief discussion is presented on current investigative efforts in the final section of this chapter. The final section of the chapter is dedicated to a brief review of Helmholtz coils and their use to generate uniform magnetic fields.

Chapter 3 covers the methodology and materials used for the investigation, beginning with cell culture methods for the CHO-K1 cell line, followed by a brief discussion about the magnetic field exposure system and exposure conditions. The chapter closes with characterization methods of the nanoparticles used and their attributes, MTT assay methodology, and finally confocal and fluorescent microscopy methods.
Chapter 4 provides information related to the results of the investigation, where the MTT assay results, as well as the confocal and fluorescent microscopy results are presented and discussed.

Chapter 5 concludes the thesis with a summary of the investigation, and provides suggestions for further development for studies of the mechanisms behind the cellular effects of these exposures, and the potential future applications of the research.
CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

This chapter briefly covers the results of a survey of relevant literature on modern research efforts directly related to cellular interactions with nanoparticles, magnetic fields, and concurrent nanoparticle-magnetic field exposures, and the mechanisms behind those interactions. Following the review discussion, a brief description of Helmholtz coils is given, along with relevant information for understanding magnetic field exposures used for this experiment.

2.1 Nanoparticle Toxicity and Cellular Interactions

Rapid development of nanotechnology has resulted in nanomaterials being incorporated into every day products and applications across many fields. Due to the unique properties of nanoparticles, they have become very attractive for many wide-spread applications, including, but not limited to commercial, medical, electronics, and military applications. Naturally, with an increase in use and application, comes an increased risk of incidental or unintended exposure. The reality of nanoparticle exposure is that everyone is exposed, all the time, as particles are now found in food, beverages, clothing, cosmetics, and various other daily-used items.

For the most part, these particles have little to no effect on health, however a growing number of studies have shown that nanoparticle interactions with cells have the ability to influence some of the most basic of cellular functions, such as proliferation, metabolism, and even cell death. As nanoparticles are very small, on the order of $1 \times 10^{-9}$ m, some nanoparticles
have the ability to permeate through biological barriers, disrupt normal functions, and cause substantial damage to biological systems. Several diseases have been associated with nanoparticle-interaction and interference with basic cell processes, such as cancer, neurodegenerative disease [28], and even Alzheimer’s disease [41]. Many investigations have thus been performed to ascertain information about the potential risks associated with various nanoparticle exposures [27–30,32,33,42] and their biological effects. For example, Hussain et al. [39] reported on the in-vitro toxicity of various nanoparticles and related concentration dependence, in BRL 3A rat liver cell model. Braydich-Stolle et al. [32] performed a similar investigation in mammalian germline stem cells. Prabhu et al. [33] studied the size and concentration dependence of cytotoxicity of Cu nanoparticles, and Comfort et al. [40] reported that superparamagnetic Fe₃O₄ particles produced a strong alteration in epidermal growth factor (EGF) activated gene transcription, with targeted genes influencing cell proliferation, migration, and receptor expression in A-431 cell line model.

Magnetic nanoparticles have become particularly of interest, due to their unique properties when exposed to magnetic fields; superparamagnetic iron oxide is already widely implemented in the medical field as a contrast agent for MRIs. Bae et al. [43], Singh et al. [30], and Mahmoudi et al. [27][42] have all reported findings on the cytotoxicity of magnetic/superparamagnetic nanoparticles, and show high biocompatibility, in agreement with the previously mentioned studies. All of these investigations together, have shown a multitude of ways in which nanoparticle exposure can influence biological systems based on the various properties of those particles, including size, shape, surface chemistry, chemical composition, exposure conditions, and even synthesis methods [44–46].
With respect to nanoparticle-cell interactions, the size, shape and surface chemistry are readily apparent as important factors that determine cellular response and uptake. Jiang et al. [47] showed that dependence of cellular interaction on size and surface chemistry, which corroborate similar reports by several others [48–51]. The reports on size dependence provide insight into how the particles interact with the cell membrane; quantum dots, and very small particles on the order of 10nm, appear to coat the plasma membrane before incorporation into the cell, and must reach a critical threshold density to trigger the cell receptors to initiate uptake, while larger particles were seen to be internalized immediately, without accumulation on cell membranes. Likewise Jiang et al. provided their report on protein adsorption layer impact on uptake showing results, similar to those by Nativo et al. [52] and Labhasetwar et al. [53], that suggest surface modification strongly modulates cellular uptake. Finally, Kim et al. [54] reported the role of cell cycle on nanoparticle uptake, and dilution amongst replicating cells; little to no impact was reported within the first 10 hours of exposure to nanoparticles. However, after 24 hours, concentration of nanoparticles in cells could be ranked with respect to cell phase. An important finding in this study was that nanoparticles that are internalized by cells are not exocytosed, but rather are split between daughter cells through cell division. Other studies, such as the investigation by the time and space resolved uptake studies by Shapero et al. [55] have reported negligible amounts of exocytosis through nanoparticle studies as well. Jiang et al. [47] published a contradictory report on the endo- and exocytosis of quantum dots, showing the particles being trafficked towards the peripheral regions of the cells for exocytosis, though this may be a result of the very small size of the quantum dots, relative to the particles used in Shapero’s studies. Ultimately, these contradictory reports reiterate the many variables
that influence cellular-level interactions with nanoparticles, and that there is still much room for improvement in understandings of the mechanisms driving those interactions. In 2007, Buzea et al. [28] published an extensive review covering the various sources and toxicities associated with nanoparticle risk and exposure. Despite the extensive research efforts conducted on nanoparticle interactions with biological systems, the true impact of these particles, and all associated effects are yet to be clearly established or well understood. Due to the relative ease by which nanoparticles can enter the human body, be it by inhalation, gastrointestinal, or skin exposure, cytotoxicity of nanoparticles has grown to be a major concern, and will continue to be so, with the constant increase in developments, and thereby demands of nanoparticle applications.

2.2 Magnetic Fields and Cellular Interactions

In modern physics and electrodynamics, electromagnetic waves are described and ordered by their frequency (f) or wavelength (λ), related by the equation $\lambda = c/f$, where c is recognized as a universal constant, the speed of light in a vacuum ($2.998 \times 10^8$ m/s). Direct current (DC) electromagnetic fields are static electric or magnetic fields which have emission frequencies of 0 Hz. While most real-world exposures will occur over a large range of the electromagnetic spectrum, much research has been conducted to investigate cellular level effects of static fields, and fields of specific frequency levels relevant to electronic appliances and power-line distributions. The magnetic fields investigated in this thesis are in the extreme-low frequency range of 0-300Hz; induced by current flowing through paired coils of wire at the respective driving frequency. This is further discussed in the methods and materials section.
Due to rapid developments in technology and electronic communications since the late 19th century, and the industrial revolution, exposure to low frequency magnetic fields is, in general, an unavoidable aspect of modern life. However, in 1979, Wertheimer and Leeper reported a correlation between living near power distribution lines, and increased risk for childhood leukemia [11]; since their report, the concern for possible health effects of low frequency magnetic field exposures has been a rather controversial topic, and the subject of many investigations. In a paper published in a section of the Journal of Electroanalytical Chemistry, Glaser [56] suggests a hierarchical view must be considered for magnetic field interactions with biological structures, in the order of atomic, molecular, cellular, and organism interactions. Glaser suggests that effects are possible on the basis of quantum mechanics, dipolar orientation of molecules, double-layer structures and modifications of systems of ionic equilibrium. Additionally, Glaser points out that, though many phenomena of field effects have been experimentally verified, the underlying effects are still not understood. This experiment focuses on the cellular level interactions of magnetic field exposure, with the consideration that many of the cellular level effects are likely not directly-driven by the field exposures, but rather by molecular-level effects.

Similar to the complexities introduced when investigating cellular-level interactions with nanoparticles, many complexities are introduced when considering cells under magnetic field exposure and their related interactions. Conflicting results have been reported with respect to cellular interactions with magnetic fields between different cell types, field exposures, and even cells of the same type, based on age or conditions [6]. The complexity of the interactions is somewhat exemplified by the proteomic analysis performed on SH-SYSY neuroblastoma cell
line model by Hasanzadeh et al. [14], where they reported 189 individual proteins altered by a 2.0mT 50Hz magnetic field exposure for 3 hours, most of these protein alterations were associated with the cellular defense mechanisms. Tenuzzo et al. [57] also demonstrate this complexity with their analysis of 6mT static magnetic field influences on apoptosis of lymphocytes, where it was reported that the effects are likely not a direct result of the magnetic field acting on the cell, but rather through Ca$^{2+}$ influx in the cells. These results were complimented when Fanelli et al. [37] reported increased cell survival by magnetic field (up to 6mT) induced Ca$^{2+}$ influx inhibition of apoptosis in several cell systems. However, contradictory evidence is presented by Ahmadianpour et al. [25] in their report of 6mT magnetic field-induced apoptosis, and altered cell cycle of p53 mutant Jurkat cells. Goodman et al. [9,58] showed an increased expression in several transcripts in HL60 cells under various types of magnetic field exposures (various frequency and field intensities), and mRNA levels after only 20 minutes of exposure. Initially, they reported that the strongest responses were measured after exposure to 1.5mT 50Hz magnetic fields; however, this same group reported conflicting data showing that the strongest responses were actually at a much lower field intensity of only 5.6μT, and that stronger fields, and longer exposure times actually lead to a reduced cellular response.

The effects of magnetic field interaction with cells have been reported to have a multitude of impacts beyond those previously mentioned, and the complexity intensifies; some reports have shown opposite effects in the same cell, with simple variations in field strength [1,20,59] while others have shown completely contradictory reports [60] compared to other researchers. Luukkonen et al. [18] reported that SH-SYSY cells, pre-treated with 100μT 50Hz
magnetic fields experienced enhanced menadione-induced DNA damage, and micronucleus formation, but alternatively also experienced an increase in DNA repair rate. Simkó et al. [5] also reported on micronuclei formation, showing yet another difference in cell line response, as they reported findings on micronuclei formation in SCL II cell line, but not in human amniotic fluid cells, after exposure to 0.1mT – 1.0mT 50Hz magnetic fields for 24 to 72 hours. The SH-SY5Y cell line has been of particular interest in magnetic field-cellular level interactions, as SH-SY5Y cells are often used as in vitro models of neuronal function; Falone et al. [15] reported increased antioxidant defenses and a shift in redox homeostasis in these cells after exposure to 1.0mT 50Hz magnetic fields for 5, 10, and 15 days, with increase in protein expression changes related to cellular defense mechanisms, similar to the study by Hasanzadeh mentioned earlier. Cerrato et al. [16] reported an increase in proliferation immediately following 24 hours of 2.0mT 50Hz magnetic field exposure with SH-SY5Y cell line, in addition to immediate up-regulation of TAU mRNA gene expression, and Syp gene expression, and immediate down-regulation of MAP2 gene expression. All three of these expressions were regulated back to normal 24 hours after exposure had ended. This indicates that some effects of magnetic field exposure may not be readily apparent in all investigations, if there is a prolonged delay between field exposure and analysis. In the studies by Cerrato et al. and Falone et al., it was also reported that a 2.0mT 50Hz magnetic field had an impact on the cell morphology, migration, and distribution of microtubules. Falone et al. observed that the cells would cluster together during culture process, but when exposed to magnetic field, would be observed evenly distributed about the culture surface. Cerrato et al. observed that the microtubules were branched radially from the perinuclear region of the control cells, while exposed cells exhibited
microtubule distributions organized into clusters that would transition into the neurites that were forming. This could indicate an effect on the microtubule structures themselves, or on molecular processes that regulate this process in the cells. Complementing these reports on morphological change in the cells, Pozzi et al. [13] reported that 2mT 50Hz magnetic fields interfere with the growth process of Lan-5 neuroblastoma cell line, and induces changes in cell morphology.

Santini et al. [61,62] have published a review article on investigations of cellular effects of low frequency magnetic fields. A general conclusion that can be drawn is that despite the multitude of studies, there still remains debate over what, if any, health effects result from exposure to low frequency magnetic fields.

2.3 Concurrent-Exposure Cellular Level Effects and In-Vitro Studies

While extensive research has been conducted to investigate the independent impacts of nanoparticle and magnetic field exposures on biological systems, studies for the combined effects of these exposures, especially at very low magnetic field frequencies are not yet well established. Current research dedicated to the combined effects of nanoparticle and electromagnetic field exposures on cells is mostly focused on effects induced at very high power absorption frequencies (kHz-MHz range) [63–66] for the purpose of thermal therapies and targeted heat generation, neglecting the lower frequency domain. A review article by Mamiya [67] on magnetic nanoparticles and targeted hyperthermia provides a brief description of particle response to AC magnetic fields for the purpose of these therapies.
As research on the combined effects of low frequency fields and nanoparticles on cells is beginning to grow, a noticeable trend is how closely related the investigations are to the independent-exposure investigations. For instance, Jia et al. [68] recently reported in 2014 the combined effects of 50Hz magnetic field and magnetic nanoparticle on proliferation and apoptosis of PC12 cells. This is not far removed from the report by McFarlane et al. mentioned in the previous section. Jia et al., however, showed data representing no statistically significant difference between control groups with and without magnetic fields, but reduced cell viability, and increased apoptosis with the application of the magnetic field to nanoparticle loaded cells, compared to cells with nanoparticles, but no applied magnetic field.

Bae et al. [43] produced a report on the effects of static magnetic fields on aggregation and cytotoxicity of magnetic nanoparticles, demonstrating that static magnetic fields induce aggregation of superparamagnetic iron oxide particle clusters, and also reduced viability, apoptosis, and irregularity of cell cycle of hepatocytes in vitro and in vivo. In this report, it was noted that the particle aggregates were the primary source of the cytotoxicity, not necessarily an interaction with the magnetic field after uptake. As well, the apoptotic effects were dependent upon particle uptake, and related reactive oxygen species production, but cell viability measurement was not. Finally, it was also reported that multinuclear giant cells became more prevalent under concurrent exposures, after long-term observation, as compared to the control groups. Shen et al. [69] demonstrated increased particle uptake with the application of magnetic field, as well as capabilities of lysosome and magnetic nanoparticle relocation by the application of external magnetic field gradients generated by two antiparallel permanent magnets. Additionally, they demonstrated the capability of these gradients to
impact cell migration by controlling the magnetic particles after uptake. The cells were observed to move along the direction of the applied magnetic field gradient, and little to no cytotoxicity was reported. The authors propose the method presented as a novel way to study cellular mechanics and intracellular forces. Increased cellular uptake of superparamagnetic nanoparticles induced by magnetic field gradient was also reported by Prijic et al. [23] showing significant increase in uptake in several different cell lines by the application of two different magnets with surface flux densities of 60mT and 403mT, and magnetic gradients of 5.5 and 38 T/m respectively, reporting that the stronger magnets were significantly more effective for impacting the internalization process; the cellular uptake rate of the particles was cell type, and time dependent. The authors also point out that the malignant cells tested had significantly higher uptake rate than the normal or healthy cells tested, as in agreement with other research referenced in their report. Comfort et al. [24] investigated the effects of concurrent 24 hour exposure to 0.5mT and 30mT static magnetic fields and gold or iron oxide nanoparticle on the HaCaT cell line. Contradictory to the report by Bae et al., the application of external magnetic fields from permanent magnets were reported to reduce, or counteract the stress response of the cells due to nanoparticle exposure, through a reduction in reactive oxygen species production, and modified gene regulation induced by the magnetic fields. These effects were observed independent of nanoparticle exposure.

Some more adventurous research has been conducted with attempts to take advantage of the magnetic properties of some nanoparticles applied to biological systems, such as González et al. [70] reporting their success in controlling magnetotactic bacteria through the application of magnetic field gradients generated by Helmholtz coils and nanoscale permalloy
islands. The authors report successful manipulation of magnetotactic bacteria, and the ability to
guide the bacteria to desired locations. Dobson [71] has reported progress towards binding
magnetic nanoparticles (100nm – 2.7μm) to cell surfaces, for the purpose of manipulating
cellular function and behavior by shear stress applied by magnetic force-actuation, while giving
a brief overview of nanomagnetic actuation for targeting ion channel activation and cell
membrane receptors. By binding nanoparticles directly to ion channels, Dobson reports success
in activating single ion channels, without interfering with normal function in other membrane
channels. Finally, Zhang et al. [38] have reported on the use of dynamic or moving magnetic
fields of very low frequency (10-40Hz) to control nanoparticles in order to enhance cellular
uptake, and then for targeted cell death by forced-lysosome disruption in both rat insulinoma
tumor cells, and human pancreatic beta cells. Lysosomal disruption was accomplished through
the application of LAMP-1 antibody coated nanoparticles, which bound to the lysosome
membranes, applying torque to the membrane by particle rotation under the influence of the
external magnetic field. The authors report that the dynamic magnetic field causes a rotational
motion in the nanoparticles, causing them to roll along the surface of cell membranes,
simulating a virus-like interaction between the particles and cells’ surfaces, thus triggering
uptake mechanisms, and enhancing internalization into the cells. These research efforts show
the potential for the ever growing applications of magnetic field and nanoparticle exposures to
biological systems, highlighting the importance of further developing the underlying
mechanisms behind cellular level interaction of nanoparticles, magnetic fields, and concurrent
exposures.
2.4 Magnetic Fields and Helmholtz Coils

There have been many coil configurations proposed for the generation of a uniform magnetic field [72] using anywhere from 2 to 5 circular, or square-wrapped coils. The simplest and most common used of these designs is the Helmholtz pair configuration; a pair of circular-wrapped conducting coils, separated by a distance equal to their radius as shown in Figure 2.1. The coils are configured to have a common axis, such that, when current is flowing through them, the resulting magnetic field can be obtained by the principle of superposition. In short, for this case, the principle of superposition states that the total magnetic field produced by two or more sources is equal to the sum of these fields; if current is flowing through the coils in the same direction, the magnetic fields add together to generate a uniform magnetic field in one direction, and if the current flows in opposite directions, the fields cancel out, resulting in a

![Figure 2.1: Sketch of Helmholtz coil, with R indicating radius of coils, with the coil pair common-axis along x-axis. Arrows indicate direction of current flow.](image)
region of essentially net-zero magnetic field. The calculation of the exact magnetic field at any point in space is mathematically complex; however, when considering positions along the central axis, such as in this experiment, the calculations become far simpler. Cvetkovic et al. [73] provide a generalized theory of Helmholtz coils and uniform magnetic field production, and DeTroye et al. [74] expand by providing a comparison between experimental measurements and analytical calculations of the field generated by a Helmholtz coil.

The magnitude of the magnetic field at any given point, along the axis of a single wound circular coil, with radius \( R \) is given by the Biot–Savart law [75]:

\[
B(x) = \frac{\mu_0 \cdot I}{2} \cdot \frac{R^2}{(x^2 + R^2)^{\frac{3}{2}}} \tag{2.1}
\]

where \( x \) is the distance from the center of the coil to any point along its central axis. Therefore, for two coils, placed at a distance \( d \) from one another, both carrying the same current \( I \), the magnetic fields measured at the midpoint between the two coils would be determined by:

\[
B_1(x) = \frac{\mu_0 \cdot I}{2} \cdot \frac{R^2}{\left(\left(x + \frac{d}{2}\right)^2 + R^2\right)^{\frac{3}{2}}} \tag{2.2}
\]

and

\[
B_2(x) = \frac{\mu_0 \cdot I}{2} \cdot \frac{R^2}{\left(\left(x - \frac{d}{2}\right)^2 + R^2\right)^{\frac{3}{2}}} \tag{2.3}
\]

where \( B_1 \) and \( B_2 \) are the magnetic fields from the first and second coils respectively, \( \mu_0 \) is the magnetic constant: \( \mu_0 = 4\pi \times 10^{-7} \ \frac{T \cdot m}{A} \), often referred to as the “permeability of free space”, \( I \)
is the current in the coils, in amperes, and R is the radius of the coils, in meters. Helmholtz coils, however, typically consists of two coils with n-number of turns per coil; accounting for the number of turns in the coils is rather simple, and can be done by multiplying the current term, I, by the number of turns in the coil, n:

\[
B_1 \left( \frac{d}{2} \right) = \frac{\mu_0 \cdot n \cdot I \cdot R^2}{2 \cdot \left( R^2 + \left( \frac{R}{2} \right)^2 \right)^{\frac{3}{2}}} \quad \text{(2.4)}
\]

and

\[
B_2 \left( \frac{d}{2} \right) = \frac{\mu_0 \cdot n \cdot I \cdot R^2}{2 \cdot \left( R^2 - \left( \frac{R}{2} \right)^2 \right)^{\frac{3}{2}}} \quad \text{(2.5)}
\]

by the superposition principle mentioned earlier, the total magnetic field between the coils can be described as a function of the distance from the midpoint of the coils x:

\[
B(x) = \frac{\mu_0 \cdot n \cdot I \cdot R^2}{2} \cdot \left( \left( x + \frac{d}{2} \right)^2 + R^2 \right)^{-\frac{3}{2}} + \left( \left( x - \frac{d}{2} \right)^2 + R^2 \right)^{-\frac{3}{2}} \quad \text{(2.6)}
\]

Noting that the most uniform field in a two-coil system is achieved when the distance between the coils is equal to the radius of each coil, and through use of a minor shift – defining the center of one coil at x = 0, and the midpoint between two coils becomes x = d/2 or x = R/2. By utilizing symmetric relations, the equations can then be simplified to say that the magnetic field at the center of the coil pair is equal to that of two times a single coil where the single coil B1 is now:

\[
B_1 \left( \frac{R}{2} \right) = \frac{\mu_0 \cdot n \cdot I \cdot R^2}{2 \cdot \left( R^2 + \left( \frac{R}{2} \right)^2 \right)^{\frac{3}{2}}} \quad \text{(2.7)}
\]
and the magnetic field at the midpoint $B_m$ is described as twice that:

$$B_m\left(\frac{R}{2}\right) = 2 \cdot B_1 \cdot \left(\frac{R}{2}\right) = \frac{2 \cdot \mu_0 \cdot n \cdot I \cdot R^2}{2 \cdot \left(R^2 + \left(\frac{R}{2}\right)^2\right)}$$  \hspace{1cm} (2.8)$$

which simplifies to:

$$B_m\left(\frac{R}{2}\right) = \frac{\mu_0 \cdot n \cdot I \cdot R^2}{\left(R^2 + \frac{1}{4}R^2\right)^{\frac{3}{2}}} = \frac{\mu_0 \cdot n \cdot I \cdot R^2}{\left(\frac{5}{4}R^2\right)^{\frac{3}{2}}} = \left(\frac{4}{5}\right)^{\frac{3}{2}} \cdot \frac{\mu_0 \cdot n \cdot I}{R}$$  \hspace{1cm} (2.9)$$
CHAPTER 3
MATERIALS AND METHODS

This chapter provides a discussion about the nanoparticle and magnetic field exposure methods and protocols, as well as descriptions of experimental instruments, setups, and procedure for each analysis performed, from the beginning of cell culture, to the viability assays, as well as confocal and fluorescence microscopy.

3.1 Cell Culture and Exposure Protocols

The CHO-K1 (ATCC® CCL-61™ Chinese hamster ovary, American Type Culture Collection [ATCC], Manassas, VA) cell line was maintained in Kaighn's modification of Ham's F-12 medium (F-12K Medium) supplemented with 10% volume fetal bovine serum (FBS) (ATCC® 30-2020™) and 1% volume penicillin/streptomycin antibiotics (ATCC® 30-2300). Subculture procedure followed the guidelines set forth by the manufacturer, modified slightly; cells were rinsed with Hank's Balanced Salt Solution (HBSS, Invitrogen-GIBCO, Waltham, MA), and the subculture ratio used was 1:10, rather than the recommended 1:4, or 1:8 due to the very rapid proliferation rate of the CHO-K1 cells. After the HBSS rinse, the adherent CHO cells were released from the surface of the culture flask by incubation for 5 minutes with 1mL trypsin (Trypsin-EDTA Solution, 1X (ATCC® 30-2101™), followed by dilution in 9mL full serum media. Cells were incubated at 37 °C, 5% CO₂ in a Heracell™150i CO₂ incubator (Thermo Fisher Scientific Inc., Waltham, MA), in 75 cm² cell culture flasks and 96-well plates for continued culture, and for exposure conditions respectively. The pH level of the culture media was controlled by 5% CO₂ in the incubation chamber, in combination with sodium bicarbonate concentration (1,500mg/mL)
in media. For visualization of microtubule cytoskeleton, CHO cells were transfected with a mEmerald-tubulin plasmid (a kind gift from Mr. Michael W. Davidson of the National High Magnetic Field Laboratory at Florida State University, Tallahassee, FL) using Effectene (#301425; Qiagen, Gaithersburg, MD) and maintained with G418 sulfate solution (#345812, Calbiochem, Philadelphia, PA). The stable transfection of the CHO cell line was performed by Dr. Marjorie Kuipers and provided as a generous gift from the U.S. Air Force Research Laboratories (Fort Sam Houston, TX).

For all exposures, cells were harvested, counted, and viability assessed using the trypan blue exclusion method using trypan blue 0.4% solution in phosphate buffered saline (PBS) (Corning-MediaTech, Corning, NY) in an Invitrogen Countess automated cell counter (Eugene, OR). Cells were then diluted into culture media and plated at 2 x 10^5 cells/mL into 96 well plates at 50 μL/well, giving approximately 1 x 10^4 cells per well. Six hours after plating, the solutions in each well were then supplemented with 50 μL of their respective nanoparticle-media dilute solution, providing each well with a total of 100 μL solution for the duration of the exposure(s). As pH value of cell culture media is critical to the optimal growth and development of the cell culture [76,77], the pH value for each dilution was measured using an Orion™ 2-Star Benchtop pH Meter (Fisher Scientific, Waltham, MA) to have values of 7.66-7.60 from complete cell culture media, to highest nanoparticle dilution concentration, respectively. For microscopy studies, cells were plated at 1x10^6 cells/mL in poly-D-lysine coated glass bottom, γ-irradiated, 35mm petri dishes ((Cat# P35GC-0-10-C, MatTek, Inc., Ashland, Massachusetts) with an initial volume of 1.5 mL cell culture media, followed by the addition of 1.5 mL dilute nanoparticle-cell culture media solution six hours later. This is a similar protocol as used for the 96-well plates.
with magnetic field, nanoparticle, and combined exposures. Immediately following the addition of nanoparticle solution, the wells and dishes were placed into the exposure chamber, to begin magnetic field exposure. Analysis of cells was then performed after 24 hours of continuous exposure. Cytotoxicity of the exposures or cell proliferation and viability were analyzed using a calorimetric MTT assay; morphology, microtubule structure, nanoparticle uptake and particle trafficking were observed via confocal microscopy. Throughout confocal microscopy, a custom buffer solution (pH 7.4, ~290 mOsm) consisting of 2mM MgCl₂, 5 mM KCl, 10mM 4-(2-hydroxyethyl)-1-piperazinee-thanesulfonic acid (HEPES), 10 mM Glucose, 2 mM CaCl₂, and 135 mM NaCl (Sigma-Aldrich, St. Louis, MO), was used to maintain the cells.

3.2 Magnetic Field Exposure and Dosimetry

The cell culture plates were placed in the center of a SpinCoil-7-X Helmholtz coil system purchased from MicroMagnetics (Fall River, MA). Driving current was supplied, and easily regulated (from 110V 60Hz line-source) by a variable-voltage, Figure 3.1: Components for magnetic field system: (a) SpinCoil-7-X Helmholtz Coil, with LakeShore probe (b) LakeShore 425 Gaussmeter (c) Kikusui PCR500M Compact Power Supply
variable-frequency Kikusui PCR500M Compact AC Power Supply (Kikusui Electronics Corporation, Yokohama, Japan) shown in figure 3.1, allowing for precise control of the system (0-270VAC, DC, 40-400Hz). A Helmholtz coil was selected as the desired magnetic field generation configuration due to the mostly-uniform magnetic field generated over a reasonably large area at the center of the coils [73,74,78]. The Helmholtz coil was placed inside a NuAire Autoflow CO₂ Water-Jacketed Incubator (NuAire Plymouth, MN), maintained at 37 °C, 5% CO₂, and power-supply run through an insulated port in the side of the chamber. The magnetic flux density was measured at multiple points across the area through which cell culture plates were exposed, using a Lakeshore 425 Gaussmeter and LakeShore Fiberglass Transverse Probe HMNT-4E04-VR (LakeShore Cryotronics Inc Westerville, OH). The Helmholtz coil was powered for one hour before cell culture-exposure began, allowing any heating and resistive change in the circuit to occur prior to executing the experiment. There was no measurable temperature rise within the exposure chamber, or region near cell culture during operation of the Helmholtz coil system, however there was a minor change in current measured through the coil after 15-20 minutes of operation. The power to the coil was briefly cut off when inserting and removing each cell culture dish, to prevent any unintended magnetic field effects on the particles as they moved into, and through the field.

In all experiments reported, field intensity was measured at 2.0mT (rms), uniform to within ± 4% over the cell exposure area, with DC, 50Hz, and 100Hz driving current for each respective experiment. Finite Element Method Magnetics (FEMM 4.2) and Comsol Multphysics 4.3 software was used to simulate the magnetic field and flux density generated by the Helmholtz coil under these conditions shown in figures 3.2 and 3.3.
Figure 3.2: FEMM simulation: Flux density plot for cross section cut of magnetic field produced by Helmholtz coil pair

Figure 3.3: Flux density measurement from center of Helmholtz coil along either X or Y axis as depicted in Fig. 3.2.2
Magnetic field parameters of 2.0mT, DC and 50Hz fields were selected because, as referenced earlier, they have been frequently investigated for impact on cellular behavior [13–16,23,25,60,79], and because 50Hz and 60Hz are common frequencies at which electric power distribution lines operate worldwide [11,80,81]. Additionally, a DC field was selected to provide insight about field interactions with the cells and particles, as compared to alternating frequency effects. 100Hz frequency was selected to determine any specific frequency dependent or frequency specific effects between 0Hz (DC), 50Hz, and 100Hz exposures. In addition, the 100Hz frequency experiment was selected in order to gleam insight towards the magnetic field-nanoparticle interactions, and to investigate if a higher frequency magnetic field would have an increased effect on nanoparticle uptake, and cell survival. Based on the initial study for 50Hz magnetic field, it was conceived that the magnetic field was interacting with the particles such that it was causing a vibratory motion, causing the particles to force their way into the cells, or somehow enhancing the cells’ ability to interact with the particles.

For all exposure conditions, a respective parallel sham experiment was performed and used as a control group. Shams were placed in the same incubation chamber as the magnetic field-exposed cells, at a distance such that the magnetic field generated by the Helmholtz coil was negligible. This was possible due to the $1/r^2$ relation of magnetic field strength, where $r$ is the distance from a magnetic field source; the magnetic field strength falls off very rapidly away from the source of the field, and therefore only a short distance outside of the coil system, the magnetic field strength is negligible. This procedure worked to ensure that any potential variability between additional environmental strain between the exposed group, and the control group was related specifically to the magnetic field exposure, and
3.3 Nanoparticles and Characterization

Two sets of commercially available iron oxide particles were purchased for this experiment, 10nm Fe₃O₄ particles dispersed in double-distilled water at a concentration of 5mg/mL from Cytodiagnostics (Burlington, Ontario, CA), and Rhodamine-B labeled 10nm Fe₂O₃ particles dispersed in DI water at a concentration of 1 mg/mL (Fe) from Ocean NanoTech (Springdale, AR). The non-labeled particles were characterized through transmission electron microscopy (TEM) dynamic light scattering (DLS) and zeta potential. Dynamic light scattering is a non-destructive method for measuring size and size distribution of particles in a colloidal solution. Zeta potential measurements can be used as an indicator of electrostatic repulsion between the particles in a colloidal solution; the greater in magnitude the zeta potential measurement, the more stable the particles in the colloidal solution should be. DLS, Zeta Potential, and polydispersity index (PdI) measurements were all taken using a Zetasizer Nano (Malvern Instruments Ltd, Malvern, Worcestershire, UK), system at the University of Texas, San Antonio, with gracious assistance from Ms. Samantha Franklin. The polydispersity index (PdI) is a measure of particle and agglomerate size distribution. Low PdI values indicate a very small size distribution, while larger numbers indicate a very large size distribution. Very large values, from 0.7-1 indicate that DLS may not provide very accurate measurements of particle size or hydrodynamic diameter, due to the large size distribution of particle agglomerates.
TEM imaging, shown in figure 3.4, was performed with a 120kV PC Hitachi H-7600 controlled TEM (Hitachi Ltd. Chiyoda, Tokyo, Japan) and was provided courtesy of Elizabeth Maurer-Gardner (Research Scientist, Materials Characterization Specialist AFRL, Dayton, OH).

Particle size was observed to be $10.97 \pm 0.70$ nm, based on the measurement of 50 independent nanoparticles. DLS and zeta potential characterization was performed for samples of nanoparticle-water, and nanoparticle-media dilutions at concentrations of 50, and 200 μg/mL in water, and 50, 200, and 500 μg/mL in media, under conditions with and without 24 hour DC magnetic field exposure. These dilutions were chosen to get an understanding of how the concentration of the particle dilution impacts agglomerate behavior under various conditions. Based on other scientific reports, it was expected that the particles would
agglomerate, due to protein-particle interaction in cell media, and the particles’ induced magnetization when subject to the application of a large external magnetic field [36,45,82–86].

The zeta potential of the particles dispersed in water was that of a moderately stable colloidal solution, with magnitude ranging from 16.2 to 38.2 mV, however the PdI was very high for the lowest concentration of particles both with and without magnetic field exposure, at 0.94-0.96, indicating that the particles had begun to sediment and settle in the solution. The average hydrodynamic diameter was measured at 1248nm and 518.2nm for the 50 μg/mL concentration in water with and without magnetic field exposure respectively, indicating that the average agglomerate size is larger with magnetic field exposure, even with sedimentation.

At the higher concentration of 200μg/mL the colloidal solution appeared to be more stable, with PdI around 0.33-0.35, and average hydrodynamic diameter measurements in the expected range of approximately 20nm and 50nm with and without magnetic field exposure respectively.

For particles dispersed in media, the zeta potential was measured significantly closer to zero, or near-neutral, with magnitude ranging from 0.16 to 9.0 mV. A near-neutral zeta potential indicates that the particles may agglomerate or coagulate fairly rapidly, however the PdI measured for all but one sample, was measured below 0.7, indicating reliable size measurements, or small size distribution of particle agglomerates. The hydrodynamic diameter, PdI, and Zeta potential measurements for all samples are displayed in table 3.1 for all particle dilution conditions. Of most importance to this study, are the magnitudes of zeta potential measurements from particle-media dilution samples, which are all less than 10, with hydrodynamic diameters ranging from 50nm – 1 μm when exposed to magnetic field.
The Rhodamine-B labeled particles were used for fluorescence microscopy experiments only, and therefore were not a focus of characterization study. The manufacturer report indicates that the Rhodamine-B labeled particles have a near-neutral zeta potential, and particle size of 10nm ± 2.5nm, with size distribution of ~10%. Ultimately, this difference between the Rhodamine-B labeled particles and the non-labeled particles is not of great concern in this particular study, as all statistically significant effects were observed at very high Fe₃O₄ concentrations, where the respective measurements in media closely reflect this same behavior.

### 3.4 Cell Proliferation and Mitochondrial Function – MTT Assay

To assess cell mitochondrial function, and thereby viability or proliferation, a calorimetric MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was used (ATCC ® 30-1010K™). In the MTT assay, a tetrazolium salt reagent is added to the cell
culture, which is reduced by metabolically active cells, and converted to water-insoluble
formazan crystals, which appear dark blue or purple under inverted microscope [87]. The
microscope used for this study was the Leica DM IL inverted contrasting microscope (Leica
Microsystems Inc. Buffalo Grove, IL). After sufficient reaction time, a provided detergent
(ATCC® 30-1010K™) is added to the wells to dissolve the formazan into a solution with the cell
media. The absorbance of this solution is then measured; in this experiment a Synergy™ HT
Multi-Detection Microplate Reader (Biotek, Winooski, VT) was used. Using the aforementioned
culture methods, cells were plated at 2 x 10^5 cells/mL into 96 well plates at 50μl/well, giving
approximately 1 x 10^4 cells per well, and then exposed to each respective exposure condition
six hours later. After 24 hours of continuous exposure, the cell media and nanoparticles were
aseptically removed, and the wells were refilled with a mixture of 100μl full cell media, and
10μl MTT tetrazolium salt reagent. The cells were let sit for two hours (more than sufficient
time to observe the crystal formation under microscope) in the Heracell™150i CO2 incubator,
before 100μl of the detergent was added to each well to dissolve the formazan crystals. The
plates were then wrapped in aluminum foil, and placed on a MaxQ™ 3000 Benchtop Orbital
Shaker (Thermo Fisher Scientific Inc. Waltham, MA), at room temperature, overnight;
absorbance readings were taken the following morning. Background readings were taken from
wells that were treated identical to the experiment wells, with the exception that there were
no cells plated. These background cells allowed for analysis to consider any residual particles
that may have been left in the wells and their potential interference with the absorption
readings from the microplate reader. An effective dose (ED_{50}) was calculated to determine the
concentration of the particles that successfully reduces the cell population to a fraction of half the control value, after the exposure time is complete.

3.5 Nanoparticle Uptake and Confocal Microscopy

To observe cell morphology, microtubule formation, nanoparticle uptake and particle trafficking, with and without magnetic field exposure, the Rhodamine-B labeled nanoparticles were added to cells cultured in poly-D-lysine coated glass-bottom dishes six hours after the cells were plated. Immediately following the addition of the nanoparticle solution, the culture dishes were placed in respective exposure and sham positions in the exposure incubation chamber for 24 hours. A Zeiss LSM 710 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) with DIC40X 1.2NA objective (Carl Zeiss MicroImaging) was used in conjunction with the ZEN 2012 software (Zen 2012 SP1 Black Edition – Ver. 8.1,3,484, Carl Zeiss Microscopy GmbH) to image the mEmerald-tubulin expressing CHO cells and Rhodamine-B labeled nanoparticles. The peak excitation and emission wavelengths for mEmerald and Rhodamine-B are 487/509 and 540/625 respectively, and the pinhole was adjusted for 1μm sections for all time-series and z-stack series. For various positions on the culture dishes, multiple images were taken in time series - 30 cycles at a rate of one cycle every 12

![Zeiss LSM 710 confocal microscope](image)

Figure 3.5: Zeiss LSM 710 confocal microscope used for microscopy studies
seconds, for a total period of six minutes, to observe cell-nanoparticle interaction, intracellular trafficking, and tubulin change. Single image acquisition of CHO cells with fluorescent tubulin expression was done with a line step of one, and line average four to acquire clear images of microtubule formations. Likewise, several series of images were taken at multiple focal points from beneath the cells, to above cells, in order to compile a Z-stack profile of nanoparticle location and distribution throughout the cell. Each series was comprised of approximately 25 to 35 images, depending on cell height, with an average interval of approximately 0.5\(\mu\)m, using pinhole sections of 1\(\mu\)m to achieve Nyquist sampling. Mean fluorescence of cells exposed to Rhodamine-B labeled nanoparticles was measured and compared between those subjected to DC magnetic field exposure and those of sham exposure conditions. DC magnetic field was selected for the microscopy study based on MTT results that displayed DC magnetic field exposure to have the most prominent effect on cell viability. As microtubules have been identified as playing a significant role in vesicular intracellular trafficking [88], CHO cells with both mEmerald-tubulin expression and nanoparticle exposures were imaged after magnetic field and sham exposure conditions, in an attempt to observe any changes in tubulin-nanoparticle interaction, tubulin expression, and microtubule structure and formation. The public domain Java-based program Fiji [89] was used for independent image and image-series processing.
CHAPTER 4
RESULTS AND DISCUSSION

This chapter presents the results of the MTT viability assay and microscopy assessments for the various conditions of nanoparticle and magnetic field exposures. Each case is presented and considered, from independent conditions for the viability assay, to varying particle concentration and field effects under microscopy. A brief discussion is presented on how the data should be interpreted for the sake of this experiment.

4.1 Cell Viability: Proliferation and Mitochondrial Function – MTT Assay

The cytotoxicity of 10nm Fe₃O₄ nanoparticles was assessed by means of MTT assay, as described in the previous chapter. The measurements for nanoparticle exposure without any applied external magnetic field are displayed in figure 4.1.1, and as sham exposures in all graphs. These results closely resemble those of other reported toxicity assessments for Fe₃O₄ particles [27,39,43], showing a minor increase in mitochondrial activity at very low doses ~10μg/mL, and significant reduction at extremely high concentrations, upward of 250μg/mL and higher. Additional assays were performed for cells cultured under nanoparticle and concurrent (DC, 50Hz, or 100Hz) magnetic field, or respective sham exposure conditions. The ED₅₀ value for nanoparticle (no magnetic field) exposure was calculated at 370μg/mL, which is also in agreement with the previously mentioned studies. Statistical significance compared to controls was determined using Student’s unpaired t-test with two-tailed P value < 0.05.

MTT assay results for the various magnetic field exposure conditions showed a minor increase in viability at low Fe₃O₄ nanoparticle concentration in most trials; however the
difference was not statistically significant when compared to sham conditions. At higher nanoparticle concentration, there was observed a marked reduction in cell viability, when compared to the respective sham exposures. These results are in agreement with those presented by Jia et al. [68], on their assessment of PC12 cell proliferation and apoptosis with 50Hz magnetic field and SiO2-modifed magnetic nanoparticle exposures.

Initially, the data suggested a trend of reduced cytotoxicity with increased magnetic field frequency; the DC magnetic field appeared to have the most significant reduction on cell viability, followed by 50Hz, and finally, the least significant impact on cell viability was observed with the 100Hz magnetic field exposure. A compilation for comparison of all exposure conditions is displayed in figure 4.1.

Figure 4.1: MTT Concentration-dependent survivability curve of cells exposed to Fe\textsubscript{3}O\textsubscript{4} nanoparticles and no magnetic field
Upon further inspection and analysis, however, it was concluded that all three magnetic field exposures have the same effect on the cells; the apparent decrease in toxicity with 100Hz magnetic field exposure, compared to DC and 50Hz frequency conditions is likely the results of a small variation in dilution for those exposure conditions introduced by pipette error. This conclusion was drawn from data shown in figure 4.2-c; a graph showing the respective difference in survival from exposure and sham conditions. The 100Hz delta curve follows almost exactly the curves of the DC and 50Hz exposures, with a right-shift of one concentration value, and peaks with the same trend. The ED$_{50}$ of each exposure condition was calculated by interpolation as 370$\mu$g/mL, 217$\mu$g/mL, 237.4$\mu$g/mL, and 303.3$\mu$g/mL for no magnetic field, DC, 50Hz, and 100Hz, respectively. The differences between ED$_{50}$ for each magnetic field exposure may yet again be attributed to the right-shift in delta mentioned previously. Due to the prominence of the DC field on cytotoxicity and cell viability measurements in the initial studies, all fluorescence and microscopy studies were performed with DC magnetic field exposure conditions in an attempt to investigate the cellular physiology effects of the exposures.

4.2 Cell Morphology, Nanoparticle Uptake and Confocal Microscopy

CHO cells with mEmerald-tubulin expression were observed under live-confocal microscopy to examine cell morphology, microtubule distribution, nanoparticle uptake, and nanoparticle trafficking after 24-hour exposure to four unique conditions. Exposures were performed at 50 and 250 $\mu$g/mL Rhodamine-B labeled nanoparticle concentrations, with and without DC magnetic field. The different nanoparticle concentrations were selected to determine if there was a direct impact on cellular uptake with respect to increased particle
concentration, while exposure to magnetic field and sham conditions were established to observe any possible changes in cell morphology, orientation or directionality, and microtubule network, as a result of magnetic field exposure.

Figure 4.2: MTT Concentration-dependent survivability curves for cells exposed to Fe$_3$O$_4$ nanoparticles and (a) DC magnetic field, (b) 50Hz magnetic field (c) 100Hz magnetic field, and (d) the respective $\Delta$ from sham conditions. The greyed section represents the area beyond the ED$_{50}$ for cells not exposed to magnetic fields. Sham exposure $\Delta$ measured against trials in an independent incubator.
Bright field imaging revealed no evident change in morphology between cells exposed to DC magnetic field and sham conditions. In both cases, the cells were observed in various shapes, sizes, and formations. Many cells, as displayed in figure 4.2.1 were observed to have three extensions away from their nuclei, towards distal focal adhesion points, but with no particular favor of directionality with or without magnetic field exposure. Likewise, confocal fluorescence microscopy using the mEmerald-tubulin expression shown in figure 4.1-b and 4.2-b revealed no change in microtubule distribution or direction between exposure conditions. While there was no change in microtubule orientation, the images also give an idea of nuclear height within the cells; there are very few cells with microtubules observed underneath the perinuclear region of the cells (observed as dark circular formations toward the center of each cell), indicating that there is no change in nuclear height between magnetic field exposure conditions.

Mean fluorescence intensity (MFI) from Rhodamine-B in the cells was measured to quantify nanoparticle uptake at both concentrations, under DC magnetic field and sham exposure conditions. Cells under concurrent DC magnetic field and nanoparticle exposure were measured to have a significantly greater MFI than that of cells exposed to nanoparticles alone, shown in figures 4.3 and 4.4. At lower particle concentrations, the difference in MFI between DC field exposure and sham was much greater when compared to the difference in MFI at the higher nanoparticle concentration. Jiang, et al. have previously reported size dependent effects of nanoparticles on cellular uptake, showing that smaller particles of ~10nm coat cell membrane surfaces before triggering endocytosis, while larger particles (or in this case agglomerates) of ~100nm or larger more readily trigger uptake [47,49,90]. The drastic contrast
at lower concentration may therefore be attributed to agglomeration effects and the size
distributions observed at lower concentrations—DLS measurements of the particle-media
solutions showed large hydrodynamic diameters up to 1μm at the concentration of 50 μg/mL.
Multiple images taken over time-series showed intracellular trafficking of nanoparticle clusters;
however, no quantification method was readily available to determine if there was a difference
in the rate of trafficking between cells exposed to magnetic field exposed cells, and those not
exposed. This is discussed further in the final section of this thesis.
Figure 4.3: Select images from confocal microscopy study without DC magnetic field exposure; (a) bright field images (b) Fluorescent mEmerald tubulin expression (c) Fluorescence imaging with Rhodamine-B labeled particles and (d) Overlay results from bright field and Rhodamine-B fluorescence imaging to show particles inside the cell structures.
Figure 4.4: Select images from confocal microscopy study after DC magnetic field exposure; (a) bright field images (b) Fluorescent mEmerald tubulin expression (c) Fluorescence imaging with Rhodamine-B labeled particles and (d) Overlay results from bright field and Rhodamine-B fluorescence imaging to show particles inside the cell structures.
Figure 4.6: Mean fluorescence intensity of cells measured with, and without DC magnetic field exposure at 50 μg/mL nanoparticle concentration

Figure 4.5: Mean fluorescence intensity of cells measured with, and without DC magnetic field exposure at 250 μg/mL nanoparticle concentration
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTION

5.1 Conclusions

A study was performed to investigate the biological impact of concurrent exposure to magnetic nanoparticles and extremely-low frequency magnetic fields (ELF-MF) using an in-vitro CHO-K1 cell line model. Protocols were established for in-vitro nanoparticle, magnetic field, and concurrent exposure, specifically for DC, 50Hz, and 100Hz 2.0mT magnetic fields, and 10nm Fe$_3$O$_4$ nanoparticles. The investigation focused primarily on comparative analysis of concurrent exposures against independent controls, to investigate cell viability and recovery through mitochondrial activity measurement via MTT assay, immediately following continuous exposure for 24 hours. The magnetic fields were generated by the application of a precisely controlled driving current through a Helmholtz coil system, in an environment-control incubation chamber. In all experiments reported, magnetic field intensity was measured at 2.0mT (rms), uniform to within ± 4% over the cell exposure area. MTT assay results indicated a significant decrease in cell viability between each magnetic field exposure condition, and respective sham at high nanoparticle concentrations. It was concluded that there was little to no effect between applied field frequencies, with only minor changes in cytotoxicity, or change in cell viability measurements with each respective field. It is worth mentioning that cells exposed to the DC, 50Hz, and 100Hz magnetic fields without nanoparticle exposure did exhibit an increase in MTT measurement, as compared to their respective sham exposures; this difference is likely due to an increase in total cell number by magnetic-field related stimulus on proliferation rate. Further
investigation is most certainly required to gleam a better understanding of the mechanisms behind this effect, as magnetic field interactions with cells are still not well known.

Confocal microscopy of the cells under concurrent nanoparticle and magnetic field exposure revealed no apparent changes in cell morphology or cytoskeletal structure between applied magnetic field, and no-field conditions. Fluorescence microscopy on the CHO cells with fluorescent tubulin expression also revealed no change between exposure conditions. The absence of effect on tubulin expression is contradictory to reports of 50Hz magnetic field effect on tubulin expression in SH-SY5Y cell models reported by Cerrato, et al. [16]. This may be attributed to the difference in cell line, or exposure time; additional studies are warranted to investigate field effect on tubulin expression between cell lines.

There was a measured change in nanoparticle uptake, with regard to applied magnetic field; a higher mean fluorescence was measured in cells subject to magnetic field exposure, as compared to the respective no-field conditions. This effect was observed at both high and low particle concentrations, but was more significant at lower concentrations. These results can be used to confirm the impact of nanoparticle concentration and agglomeration effects on cellular uptake. Intracellular trafficking of nanoparticle clusters was observed with all exposure conditions; however no quantification method was available to determine if there was a difference in the rate and directions of trafficking.

5.2 Recommendations and Future Directions for Investigation

With the current investigation methods, it is not possible to know the exact method of endocytosis, or storage and trafficking of the nanoparticles once they have been taken up into
the cells. By changing the label on the nanoparticles, and systematically selecting alternative
photoactive tags for cell proteins, such as the lysosomal-associated membrane protein 1
(LAMP1), and Tetramethylrhodamine (TRITC), co-localization of the particles and lysosome
structures may be observed. Additionally, inhibitory drugs that interfere with specific pathways
may be systematically selected and administered to the cells to identify the particular uptake
mechanisms involved with these particles.

While there was no observed effect on cell viability between the DC, 50Hz, and 100Hz
magnetic fields, that is not to say that there are no proteomic or genomic changes within the
cells between these, and other applied external magnetic field frequencies. A brief investigation
into reactive oxygen species (ROS) or Interleukin 6 (IL-6) as an indicator of cellular stress and
inflammatory response, in addition to an assay to reveal caspase 3/7 activities could provide
some insight into the cause of changes in mitochondrial function reported via MTT assay. As the
mechanisms behind magnetic field and cell interactions are still not well known, further in-
depth studies could be developed to investigate individual proteomic and genomic expression
within the cells exposed to various magnetic field conditions, similar to the analyses reported
by Falone et al., and Hasanzadeh, et al. [14,15] with SH-SY5Y neuroblastoma cell lines exposed
to 50Hz magnetic fields for 3 hours.

Ultimately, there are near infinitely many variations available for experimentation from
field specifics such as frequency, intensity, and direction, along with dynamic and moving fields
or static field gradients, as well as nanoparticle size, shape, composition, fabrication method,
and time duration of each related exposure. An important and more impactful direction to
focus future efforts would be on bridging the gap in knowledge between the specific
mechanisms behind the magnetic field interactions with cells, to better understand why different cell types have different responses to the same exposure conditions, and how to take advantage of said mechanisms. Future applications of these advantages may be developed to eliminate deleterious effects, and enhance desirable or salubrious effects in medical, commercial, and military applications.
REFERENCES


