CYTOTOXICITY OF BIODEGRADABLE MAGNETIC NANOCOMPOSITE SPHERES FOR DRUG DELIVERY PURPOSES

A Thesis by

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with in a major in Mechanical Engineering.

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DEDICATION

To my parents, my brother and my dear friends

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ABSTRACT

The use of nanotechnology is growing rapidly, with potential applications ranging from production to electronics to medicine. Nanotechnology has been proven to have a great impact on biomedicine through its applications in tissue engineering, cancer therapy, hyperthermia, and other drug delivery purposes. Nanomaterials can be fabricated and manipulated to suit the requirements for a particular function. Drug delivery through magnetic nanoparticles is being used for site-specific and controlled drug-release purposes. Magnetic drug transport involves encapsulating a drug in a magnetic nanosphere and administering it intravenously to deliver it to a particular organ or a receptor for therapeutic purposes. Nanotechnology-based drug delivery maximizes patient compliance and targeting efficiency, and thus reduces the toxicity of the drug to normal cells. Nanotechnologies that are being used in medical applications for diagnostics, as drug carriers, and for prosthesis and implants have raised interest and concern about their biocompatibility and toxicity. It has been shown that nanomaterials that come in contact with the human body can affect the central nervous system and cause inflammatory responses in the lungs, liver, spleen, etc.

In this research, emphasis was placed on determining the toxicity of nanocomposite spheres made from two magnetic nanoparticles—nickel ferrite and cobalt ferrite. These magnetic nanoparticles were fabricated using a sol-gel process and then used to fabricate nanocomposite spheres using PLGA as a polymer and an oil-in-oil emulsion/solvent evaporation technique. Different samples were made with different nanoparticle compositions, and these samples were tested for cytotoxicity using a standard colorimetric test using MTT assay. Viability tests were conducted on these cells to determine the toxicity by varying the composition and concentration of the nanoparticle, and then comparing the two different nanomaterials.

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LIST OF ABBREVATIONS

ADEPT	Antibody Directed Enzyme Prodrug Therapy
BBB	Blood-Brain Barrier
CNS	Central Nervous System
CNT	Carbon Nanotube
DDS	Drug Delivery System
DNA	Deoxyribonucleic Acid
EPR	Enhanced Permeability and Retention
FDA	Food and Drug Administration
GDEPT	Gene-Directed Enzyme Prodrug Therapy
GI	Gastrointestinal
LMP	Lysosomal Membrane Permeability
MPS	Mononuclear Phagocyte System
MRI	Magnetic Resonance Imaging
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
OD	Optical Density
PAMAM	Polyamidoamine
PGA	Polyglycolic Acid
PLA	Polylactic Acid
PLGA	Poly-Lactic-co-Glycolic Acid
RES	Reticulo Endothelial System
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate

VDEPT	Virus-Directed Enzyme Prodrug Therapy
VSM	Vibrating Sample Magnetometer
XRD	X-Ray Diffraction

LIST OF SYMBOLS

nm	Nanometers
µm	Micrometers
Oe	Oersted
K	Kelvin
°C	Degrees Celsius
g	Grams
rpm	Revolutions per minute
ml	Milliliters
µl	Microliters
µg	Micrograms
mg	Milligrams

CHAPTER 1

INTRODUCTION

Nanotechnology covers many fields, ranging from electronics to cosmetics, as well as biomedical engineering for tissue engineering, gene delivery, drug delivery, MRI, and many more [1]. The feature of nanotechnology that makes it so applicable in many fields is its small size, which has advantages like high surface area and low surface defects. The medical industry has been working on cancer for the past few decades, trying to cure it with various drugs. These anti-tumor drugs must be injected into the body as they cannot be orally absorbed; ingestion would cause some toxic effects to normal cells within the body or premature clearance through phagocytosis or through the reticulo endothelial system (RES) [2]. Thus, these toxic drugs should be wrapped or enclosed in a material that works as a carrier, a carrier that is biocompatible with the cells in the body and that delivers the drug to the treatment site only. The entire process, called drug delivery, involves synthesizing drug carriers that enclose the drug and then, with careful administration, injecting them into the body. This process protects the drug and allows it to reach the target site with the help of receptors that are biodegradable and compatible with the living organism, like proteins, ligands, or antibodies. Finally, the drug diffuses from the carrier at the target site for a certain period to complete its therapeutic mechanism followed by degradation of the carrier.

Drug delivery has involved the investigation of many nanodevices, such as polymer micelles, liposomes, dendrimers, and nanoparticles. No single nanodevice is ideal for all biomedical applications. Therefore, they should be modified and synthesized in a manner that fits a specific application. The devices need to meet specific requirements to be suitable for drug delivery. For example, they should be between 100 nm and 1,000 nm in size to be able to travel

through the capillaries. The devices should also be biocompatible, biodegradable, non-toxic, non-immunogenic, and non-antibody. Another reason for entrapping the drug within the devices is that some drugs are insoluble in aqueous media.

Nanomaterials have been observed to be toxic to the body. When inhaled, they can cause inflammation in the lungs and trigger cell death. Dendrimers are nanoparticles that have been used for decades, mainly for MRI scans and drug delivery, and they can handle a very large drug load. Certain types of dendrimers are proven to be toxic and thus affect red blood cells. Increasing a drug dosage increases the molecular weight of the dendrimer, which in turn increases its size and results in rapid clearance. Liposomes are efficient for drug delivery, but they cannot carry large drug loads and cannot accommodate insoluble drugs, since they have an aqueous medium in which to carry the drug. Polymer micelles can carry the drug, but they have stability issues within an aqueous media. This thesis concentrates on nanocomposite spheres fabricated with magnetic nanoparticles for targeted drug-delivery applications.

For targeting drug delivery, one of the best methods is to use magnetic nanoparticles and direct these particles using an external magnet. These magnetic nanoparticles are widely investigated for use as MRI contrast agents, carriers for targeted drug delivery, etc. This paper explains how magnetic nanocomposite spheres were fabricated with different compositions of cobalt ferrite and nickel ferrite nanoparticles, and a cytotoxicity test was conducted using a colorimetric test with MTT to find the cell viability at different concentrations of samples.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 Nanotechnology

Nanotechnology, in combination with nanoscience, controls matter at a molecular level, with a scale less than 1 micrometer—usually between 1 and 100 nm [3]. This nanotechnology can be precisely defined as the "design, characterization, production and application of structures, devices and systems by controlled manipulation of size and shape at the nanometer scale (atomic, molecular and macromolecular scale) that produces structures, devices and systems with at least one novel/superior characteristic or property" [4]. Nanoscience is a part of nanotechnology that helps to study the manipulation of materials at atomic, molecular, and micro molecular levels, since the properties of the materials differ at these atomic and sub-atomic levels when compared to particles on a larger scale. When a material occurs in bulk, there is an average of all quantum forces acting upon its atoms; however, when the material is made smaller, these quantum forces no longer act upon the atoms, which results in a change of the material's properties. This change in material properties at the nano-level improvises the material, since it tends to have a large surface area, and the quantum effects allow it to enhance or change its properties, such as chemical reactivity, strength, and electrical characteristics [5].

2.2 Fabrication of Nanomaterials

Two approaches for synthesis and fabrication of nanomaterials are shown in Figure 1 [3].

- Top-down approach: The top-down approach introduces slicing, grinding, or successive cutting of a bulk material into nano-sized materials. This has been the dominant process in the production of semiconductors. The basic disadvantage with this approach is that it results in surface imperfection, crystallographic damage to the patterns, contamination of

the materials, and, due to the small size, heat dissipation through the material that most likely introduces internal stresses inside the particles. However, the top-down approach has the capability of producing nanomaterials in bulk [6].

- Bottom-up approach: The bottom-up approach works on the principle of self-assembly. Everything in nature is built based on a bottom-up approach, including cells, crystals, and human beings. This process starts at an atomic scale and acts by coordinating independent entities to produce ordered structures or a desired shape. This approach provides nanomaterials with homogenous chemical compositions and fewer defects [6].

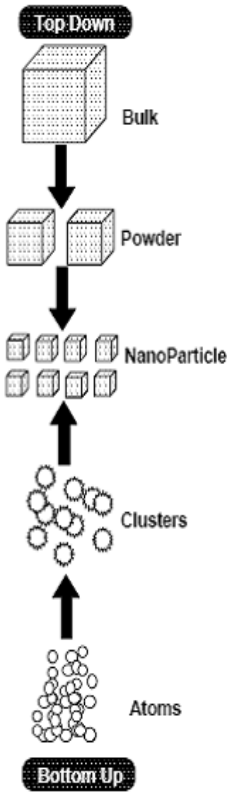


Figure 1: Top-down and bottom-up approaches to nanofabrication [3].

2.3 Classification of Nanomaterials

Nanomaterials are primarily classified based on their dimensions, morphology, composition, uniformity, and agglomeration. Figure 2 shows the classification of nanomaterials based on these characteristics [7].

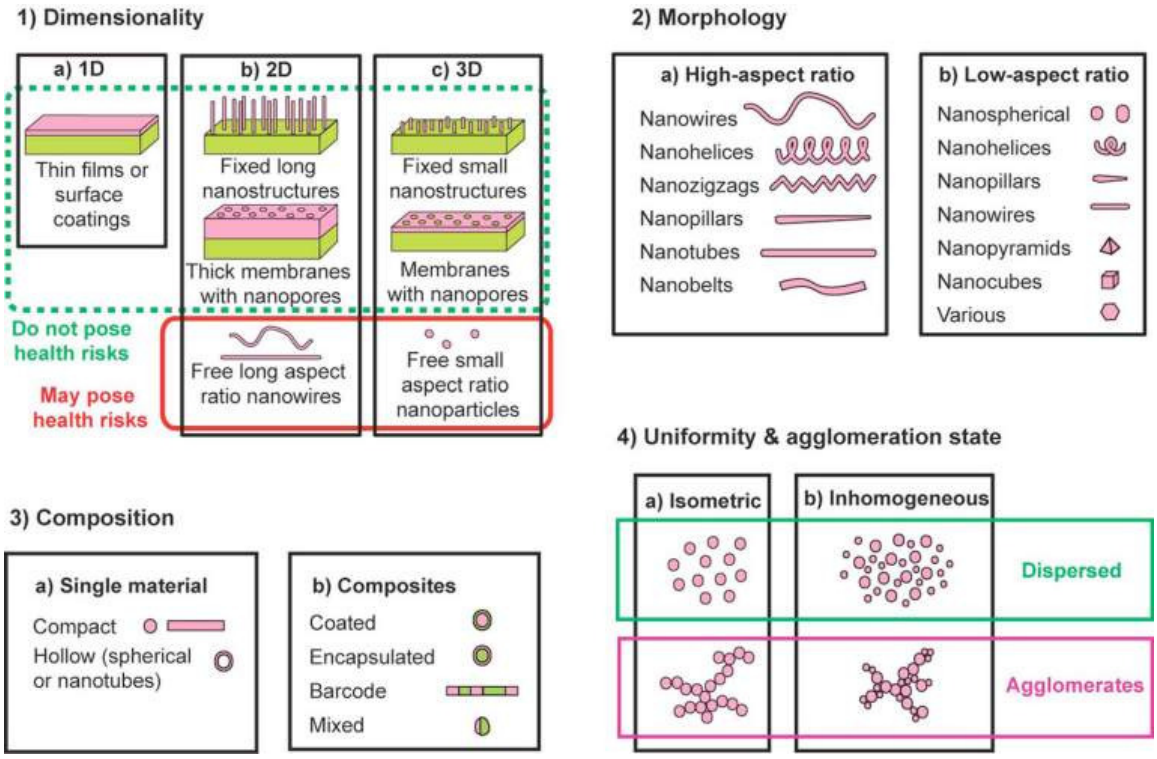


Figure 2: Classification of nanostructured materials [7].

- One-dimensional (1D) materials have one of their dimensions in nanoscale. Usually these can be referred to as thin nanofilms or surface coatings. They are grown in a controlled manner by restricting them to being one atom thick, called a monolayer, or else they can be deposited by various methods. These films are mainly used in electronic-device manufacturing, engineering, and chemistry. The properties are well understood from atomic levels and upwards, even in quite complex layers such as lubricants.
- Two-dimensional (2D) nanomaterials have two dimensions in the nanometer scale. These 2D tubes and wires are of considerable interest in research industries because of their novel electrical and mechanical properties.
- Three-dimensional (3D) nanomaterials have all three dimensions in nanoscale. These include thin films deposited under conditions that generate an atomic-scale, colloids, and free nanoparticles with various morphologies [7].

Nanomaterials possess different morphologies such as flat and spherical. Generally they are classified as materials with a high aspect ratio or low aspect ratio. Nanoparticles with a high aspect ratio include nanotubes, nanowires, etc. Carbon nanotubes (CNTs) are 2D nanomaterials, first observed by Dr. Sumio Iijima in 1991 [3]. These are extended tubes of graphene sheets with a diameter of a few nanometers and a length of several micrometers or centimeters. Carbon nanotubes can be single-walled or multi-walled. They play an important role because they are mechanically strong, flexible about their axis, and can potentially conduct well whether their chirality is semiconductor or metallic. For these reasons they are used in sensors, display devices, and reinforced composites. The uniform production of CNTs can be achieved with specific requirements. Nanowires are ultrafine wires or sometimes dots formed by self-assembly; they possess remarkable optical, electronic, and magnetic properties, thus making them potential material for use in high-density data storage, magnetic read heads, and opto-electronic devices. These nanotubes and nanowires come in different shapes, such as helices, zigzags, belts, and pillars [7]. Nanomaterials with a low aspect ratio include nanoparticles that are shaped as spheres, cubes, or pyramids, and the collection of these materials can be in powder, suspension, or colloids.

Single nanomaterials can be fabricated by various methods and can be coated in different ways to attain the desired output if being used in composites. Magnetic nanocomposites have a tendency to agglomerate, depending on their chemistry and electromagnetic properties. They can appear like clusters, suspensions, or an agglomerated state until they are coated with non-magnetic particles. Agglomerated nanomaterials tend to behave like large particles, which should be taken into account when there are concerns about health and environmental effects.

2.4 Properties of Nanomaterials

Nanomaterials have surface features that are different from atoms and their bulk materials, while most microstructured materials possess properties that are similar to their bulk materials. Therefore, due to the small size of nanomaterials, they possess the following: (1) a large surface area-to-volume ratio; (2) high surface energy; (3) spatial confinement, which alters the quantum confinement; and (4) reduced imperfections. The quantum confinement of nanomaterials has a high impact on their properties and band structure. Charge carrier density in nanomaterials can be modified differently from bulk materials, which in turn alters the electronic and optical properties of the materials. Reduced imperfections and purification can be obtained in nanomaterials by a self-purification process like thermal annealing [8].

Mechanical properties like hardness, toughness, scratch resistance, and fatigue strength are modified in size when bulk material is changed to nanomaterial. This enhancement in the mechanical properties is due to the reduction in imperfections at nanoscale. The small size makes the materials free from internal structural imperfections like dislocations, micro twins, and impurity precipitates, which occur when the impurities present in the highly energetic nanosize materials tend to travel to the surface during the purification processes. Due to the improved mechanical properties of nanomaterials, they can serve potentially as high-frequency electro-mechanical nano resonators, mass sensors, and microscope probe tips, and in macro scale applications. In polymer composites for example, nanofillers can improve the mechanical properties of the polymer materials when used in the composites. Nanomaterials can also be used as high-strength materials, flexible conductive coatings, and cutting tools. Super hard nanocomposites can be fabricated to help in the production of hard protective coatings [4].

Recent advances in experiments have proven that some nanomaterials have extraordinary thermal properties compared to their macroscopic components [6]. As dimensions are reduced to the nanolevel, the size of the particles is comparable to the wavelength and mean free path of photons. Therefore, photo transport can be significantly changed due to photon confinement, and the quantization of photon transport results in modified thermal properties. For example, nanowires made of silicon exhibit smaller thermal conductivities compared to bulk silicon, whereas carbon nanotubes exhibit extreme high thermal conductivity in axial directions, leaving high anisotropy in the heat transport of the materials. Interfaces or grain boundaries between similar materials are proven to be important, since the interface disorder scatters photons, resulting in the possibility of nanomaterial structures with high interface densities reducing the conductivity of thermal energy. Nanofluids generally referred to as solid-liquid composites, consist of nanomaterials that can be used to enhance thermal transport, thereby increasing the thermal properties of nanomaterials [9]. Figure 3 summarizes different experiments that have proven that fluids with nanomaterials increased the thermal conductivity based on the volume fraction of the particular material [9].

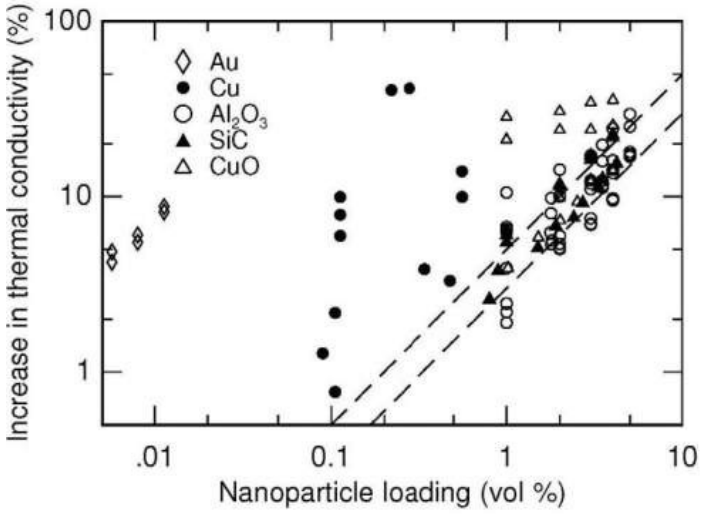


Figure 3: Relative increase in thermal conductivity as a function of volume fraction of nanomaterials [9].

2.5 Drug Delivery

An increasing understanding of the human body and the explosion of new treatments have resulted in the discovery of bioactive molecules, gene therapy, and time-release medications. Current drug delivery methods have specific issues concerning therapeutic efficiency, in particular partial degradation before reaching the desired target sites, safety and efficacy shortcomings in their conventional administration modalities, drug instability in biological environments, and premature drug loss through rapid clearance and metabolism. To overcome these obstacles, new drug delivery systems are used [2]. The goal of new drug delivery systems is to deploy medication to specific targeted parts of the body by a medium that can administer and control the release of drugs by means of physiological or chemical triggers. This technology can address current difficulties like expanding the product life or even add to their performance and acceptability by improving safety and patient compliance [2]. Hence, nanotechnology has been studied for its use in a new drug delivery system. Nanostructured drug carriers based on silicon compounds, polymers, antibodies, and magnetic particles allow the medication to be administered directly to the diseased sites, allowing more effective bioavailability at lower doses with relatively fewer side effects. Artificial nanostructures, like polymer nanoparticles of the same size as the biological matter, interact well with biomolecules on both the surface of cells and inside them [10].

2.6 Drug Delivery Systems

Different drug systems have been employed for different purposes and requirements. For example, oral drug delivery is used for fast dissolution and good gastrointestinal retention time, and to enhance absorption. Various carriers are used for drug targeting including polymer

micelles, collagens, micro- or nanospheres, nanoparticles, liposomes, and resealed red blood cells [5].

Drug delivery systems (DDSs) are formulations of a device that enables efficient drug delivery to a target site within the body [11]. They also improve the efficiency, availability, and safety by controlling the time and rate of release of drugs within the body. This drug delivery process includes different routes of administration and transport of drugs crossing biological membranes to the site of action. The therapeutic agent includes the drug, drug carriers, and sometimes a homing device for targeted drug delivery. DDS can be drugs administered for a therapeutic purpose or the delivery device used to transfer the drug to the diseased site. Novel carrier materials should possess the following [1]:

- Structural control over size and shape of drug.
- Biocompatibility and non-toxicity.
- Precise, surface modifiable functionality for all specific targeting.
- Appropriate cellular adhesion and intracellular trafficking to allow drug delivery to the cytoplasm or nucleus.
- Protection of drug release from activation during transit to target cells.
- Consistency and reproducibility.

Drug carriers must overcome certain obstacles to deliver drugs. The drug carrier should pass through the endothelial lining of the blood circulation system and certain barriers like the blood-brain barrier (BBB) [11]. The characteristics of the barriers are currently being researched, but it is clear that particulate systems larger than 100 nm cannot pass the barriers. Apart from the barriers, mononuclear phagocyte systems (MPSs) are responsible for clearance of “foreign body-like materials” and are considered an important part of the body’s immune system. Sometimes

drug carriers can be considered foreign materials and are cleared away by the MPS without actually delivering the drug to the target site. Or, sometimes lower doses might be available at the desired site. Clearance of materials by the MPS is highly dependent on particle size, particle charge, and surface hydrophobicity [12, 13]:

- Particles arranging in size from 10 nm to 7 μm are cleared away by the MPS; therefore, particles should be less than 10 nm.
- Liposomes possessing negative charges are cleared away rapidly compared to neutral charge particles.
- Hydrophobic particles are immediately considered as foreign by the MPS, for these particles must be hydrophilic to escape MPS clearance.

2.6.1 Nanocomposite Spheres

Nanoparticles coated with biodegradable polymers or drugs encapsulated within the nanoparticles matrix become hydrophilic and less vulnerable to destruction by the body's immune system [14]. As a result, they can be better absorbed and produce greater results at safer doses. Active drug particles less than 100 nm in size travel through tumor pores, and when entrapped, they can be delivered to the target sites at a reduced risk of causing adverse side effects [13].

Nanocomposite spheres are sub-micron particles, with the desired drug encapsulated, absorbed, or entrapped within the polymer matrix. Biodegradable and biocompatible polymers should be used for localized and sustained drug delivery [14]. The smaller size of nanoparticles offers several advantages over micro particles. Nanoparticles have generally high intracellular uptake compared to micro particles, and according to the studies by Panyam and Labhasetwar, 100 nm sized nanoparticles showed a 2.5 fold higher uptake compared to 1 μm micro particles

and 6 fold higher uptakes compared to 10 μm micro particles [15]. Nanoparticles can cross the BBB, following the opening of tight junctions, and with such a strategy, they can be used to deliver the therapeutic agent for difficult-to-treat diseases like brain tumors. Nanotechnology in drug delivery helps to do the following [16]:

- Enhance delivery, which leads to superior performance.
- Increase the life span of the drug by reformulating it through a novel delivery system.
- Improve patient protection.

However, nanoparticles for drug delivery may not be suitable in cases where the drugs are less potent at higher doses, thus making the drug delivery system much larger, which in turn makes it difficult to administer within the body. Biomedical applications of nanotechnology include drug delivery, imaging, tissue engineering, gene therapy, biomarkers. and biosensors [17].

2.6.1.1 Polymer Drug Delivery

Polymers have been of great interest for use in drug delivery systems. The physical and chemical properties of polymers can be controlled by molecular synthesis [18]. Drugs can be loaded into a polymer by entrapping the drug in the polymer-matrix or encapsulating it within the polymer. Traditionally, the drug is released through diffusion control. Biodegradable polymers degrade into acceptable biological compounds by hydrolysis, leaving the incorporated medication behind. The degradation process happens by breaking down the polymers, which eventually are reduced to waste that can easily be discarded by the body. Appropriate polymer selection is necessary to develop a successful DDS [10]. The polymer can be synthetic or natural and either biodegradable or non-degradable. The major drawback with natural biodegradable polymers like collagen and cellulose is their short life and inability to resist strong solvents

during the fabrication of nanocomposite spheres [10]. Non-degradable polymers should be removed from the body after drug delivery with surgery. Biodegradable polymers are being fabricated to manipulate the properties depending upon the delivery requirements. Certain factors like hydrophilic, amorphous, low-molecular-weight polymers, grown by a stepwise process or condensation reaction, can alter the rate of degradation and control the rate of drug release [18, 14].

Biodegradable polymers can be single polymeric networks or block copolymers, which are formed as a network through the combined polymerization of two or more different networks. These copolymers consist of cross-linked combinations of hydrophilic and hydrophobic monomers [14]. These monomers self-arrange themselves with their hydrophilic and hydrophobic ligands aligned on opposite sides in an aqueous medium, with the hydrophilic portions forming the outer shell. The hydrophilic outer shells protect the core and the drug from chemical degradation during travel within the aqueous solution. Further drug release occurs by polymer degradation mechanisms depending on the specific target. Synthetic polymers have an advantage of sustained release of the encapsulated therapeutic agent over a period of days to several weeks. Of all the natural and synthetic polymers, polylactic, chitosan acid, and polylactic-co-glycolic acid (PLGA) are extensively studied for drug delivery [5]. These polymers are biodegradable, biocompatible, and non-toxic, and have been used in biomedical applications for many decades [19].

The discovery and first synthetic work on low-molecular-weight forms of lactide and glycolide polymers occurred several decades ago. In 1970, polyglycolic acid (PGA) was used as a biodegradable suture material, which led to large-scale production in the biomedical polymer market [20]. Having been being approved by the Food and Drug Administration (FDA), PLGA is

being used for ligament reconstruction, tracheal replacement, surgical dressings, vascular grafts, and dental and fracture repairs [21, 15, and 14]. PLGA is made at a relatively slow rate so that it does not affect normal cell function. The biodegradation phenomenon of PLGA is important, since it determines the rate and mechanism of release of the therapeutic agent. The release of the therapeutic agent is biphasic, beginning with diffusion of the therapeutic agent through the polymer matrix and followed by the degradation of the polymer along with diffusion of the drug. The copolymers of PLGA degrade to lactic and glycolic acid, which can be metabolized by the Kreb's cycle into carbon dioxide and water [21]. The degradation process is affected by several factors including preparation method, the presence of low-molecular-weight compounds including monomers, catalysts, and the shape and size of the particles. Properties of the polymer, such as hydrophobicity, crystallinity and glass transition temperature, physiochemical parameters, and mechanism of hydrolysis also affect degradation [20].

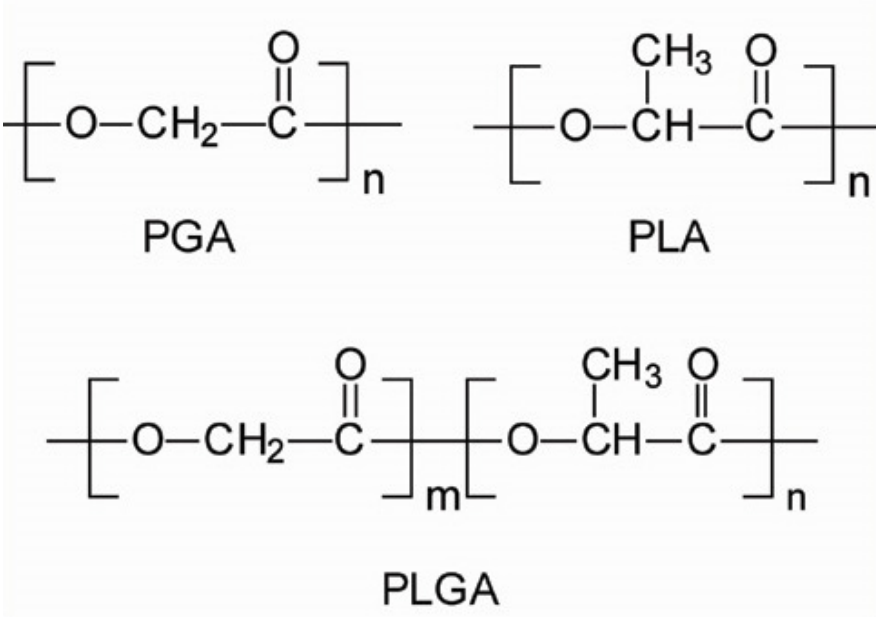


Figure 4: Chemical structure of PLGA [19].

The hydrophilic nature of the polymer accelerates the degradation of nanoparticles. The more hydrophilic the polymer, the more it degrades. The hydrophilicity depends on the ratio of

crystalline-to-amorphous regions in the polymer, which in turn depends on copolymer composition. Figure 4 shows the chemical structure of PLGA, which is formed from polylactic acid (PLA) and PGA monomers. Lactic acid being more hydrophobic than glycolic acid makes lactide-rich PLGA copolymers less hydrophilic than glycolic-rich PLGA copolymers. Other than hydrophilicity, degradation time is shorter for polymers with low molecular weight and more amorphous polymers [10]. The most widely used composition of PLGA is 50:50, which degrades within 50 to 60 days, whereas D, L-lactide/glycolide compositions of 65:35, 77:25, and 88:15 have comparatively longer lifetimes. Poly (D, L-lactide) needs 12 to 16 months to degrade completely, whereas poly (L-lactide), being more crystalline, takes 1.5 to 2 years to degrade completely [20].

Toxicity and biocompatibility are two critical issues to be considered while selecting a material for the design and development of nanoparticles for pharmaceutical formulations [12]. Biocompatibility can be considered the capability of the material to perform the required host response at a specific application. This host response should result in minimum tissue damage and in turn support the regeneration process [22]. Two main factors that determine biocompatibility are host reactions induced by the material and degradation of the material within the body. Hence, the calculation of degradation time along with local tissue clearance are important for choosing the drug and polymer concentration in the tissue [22, 10].

2.6.1.2 Properties of Cobalt Ferrite Nanoparticles

Cobalt ferrite is a well-known hard magnetic material. These nanocrystalline magnetic powders have many applications as ferrofluids in magnetic drug delivery, and in hyperthermia for cancer treatment [23]. The observable properties of cobalt ferrite include high saturation magnetization, high coercivity (10.2 KOe), strong anisotropy, along with good mechanical

hardness and chemical stability. The particle size and size distribution of cobalt ferrite prepared by the sol gel method are dependent on annealing temperature and annealing time [24]. An experiment was conducted by Toksha et al. to observe the effect of annealing temperature and annealing time on the size of particles. In this experiment, the particle size was calculated using the Scherer formula, with the most intense peak (311) observed from a XRD pattern. The initial particle size was observed to be 15 nm when calcined at 570°C. By annealing the particles at 800°C and 900°C for about 10 hours, the sizes of the particles were increased to 24 nm and 28±2 nm. Further annealing to 1000°C for 8 hours and 12 hours increased the sizes to 32 and 40±2 nm. Based on these results, a graph was plotted to demonstrate the increase in the particle size, as shown in Figure 5. The graph explains the increase in particle size with a rapid increase of the annealing temperature between 700°C and 900°C and a slow down after 900°C. A long annealing time enhances coalescence, resulting in larger particles, which explains that the size of the nanoparticles produced by the sol gel technique can be controlled by annealing time as well as annealing temperature [24]. Another comparison was made to observe the magnetic behavior in response to increase in particle size, and conclusions can be drawn based on Figure 6 [24].

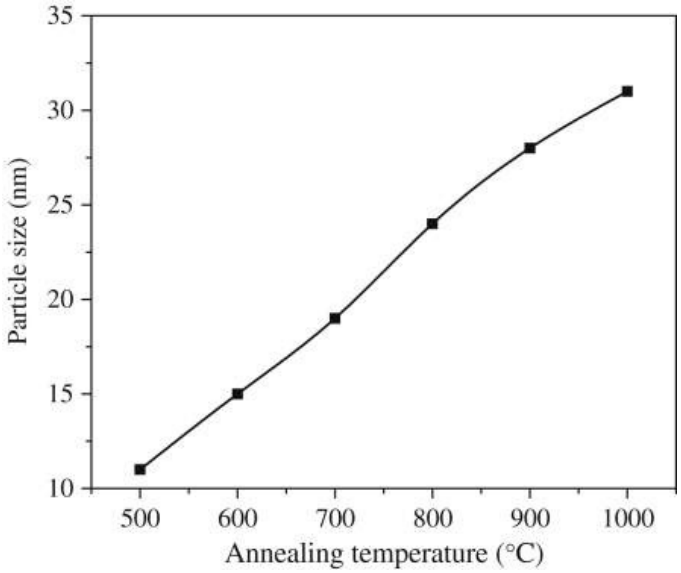


Figure 5: Variation of particle size with annealing temperature [24].

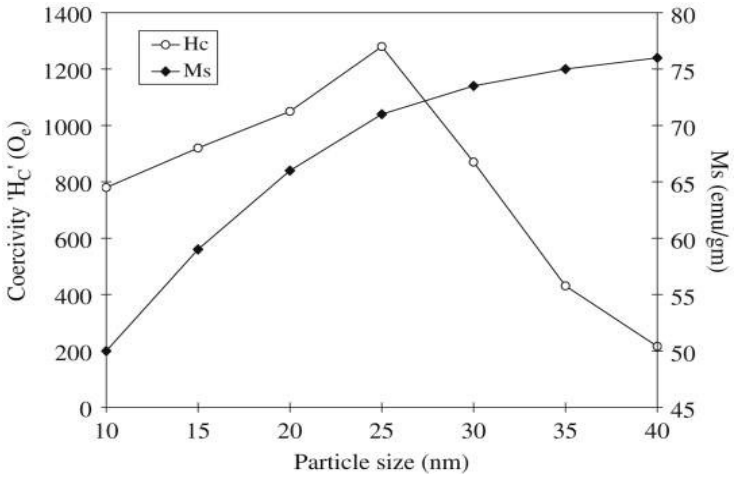


Figure 6: Variation of H_c and Ms with particle size at room temperature [24].

Magnetic characterization was determined using a vibrating sample magnetometer (VSM) with a maximum applied field up to 12 KOe. For the particles with 15 nm at room temperature, the coercivity was 1215 Oe, and at 77 K, the maximum coercivity of 10.2 KOe was noted. From this it is clear that the coercivity has a maximum value at 25 nm, and with an increase in size, the coercivity decreases, but the saturation magnetization was observed to increase with the annealing time [24].

2.6.1.3 Properties of Nickel ferrite

Nickel ferrite has been of great interest for biomedical applications, especially for magnetic drug delivery and hyperthermia for its biocompatibility [25, 26]. It has relatively low toxicity, large anisotropy with moderate magnetization, high coercivity, and high chemical and structural stability [27, 28]. Nickel ferrite nanoparticles prepared by the sol-gel process exhibit an inverse spinel ferrite structure, and there was some aggregation observed due to the magnetic attraction, which affects the size and the shape of the nanoparticles [29]. It was observed that oxidation time affected the size of the nanoparticles. It was also observed from the XRD patterns that an increase in the oxidation time increased the sharpness of the peaks. The XRD pattern from Figure 7 shows the most intense peaks from the oxidation times of 180 and 30 minutes

(311), which when calculated using Scherer formula gives particles sizes of 26 nm and 38 nm. An increase in the oxidation time changed the shape of the nanoparticles from a spherical to cubic spinel structure [28, 30].

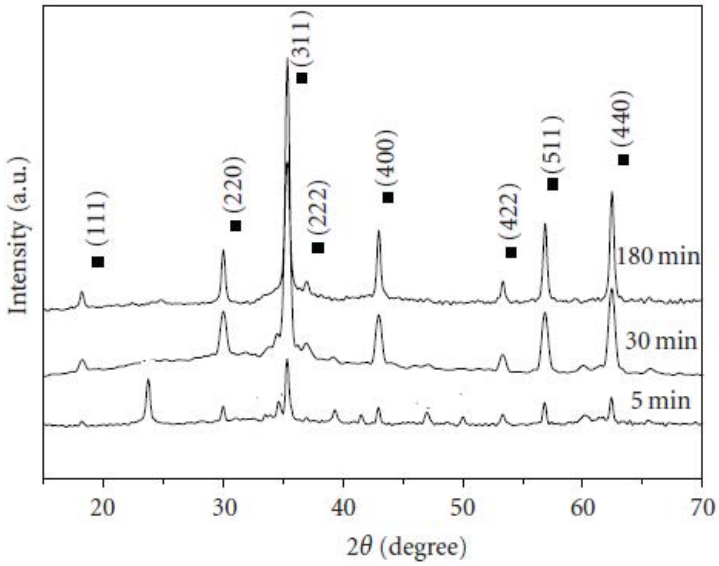


Figure 7: XRD pattern of $CoFe_2O_4$ nanoparticles [30].

The saturation magnetization (M_s), and coercivity (H_c) increased with the increase in the oxidation time, but when compared to the oxidation times of 180 and 30 minutes, there were slight fluctuations in the readings, which can be due to small differences in particle size, thus indicating that the influence of these differences has a low effect on the magnetic behavior [30]. The saturation magnetization value of particles at a size of 38 nm was 63 emu/g, coercivity was 944 Oe, and remanence ratio was 0.40, which decreased with an increase in the oxidation time.

2.6.2 Polymeric Micelles

Polymeric micelles are amphiphilic nano-sized carriers made from polymers by self-arrangement within the aqueous solution. Self arrangement is mainly due to ionic or hydrophobic interactions between the polymer segments [16]. Polymeric micelles have a core-shell structure—the core consisting of a hydrophobic or ionic part with molecules of a water insoluble drug and the shell, which are hydrophilic, providing interactions with the aqueous environment

making its structure favorable for drug delivery. Polymeric micelles can be made with block copolymers or graft polymers. Block polymers are usually linear polymers composed in a sequence with at least two or more polymers having different physico-chemical properties. Graft polymers are side chain segments attached to the main polymer chain [31].

Good thermodynamic stability in a physiological solution and low micellar concentration makes the use of polymeric micelles more promising than regular surfactant micelles. Certain advantages of polymeric micelles that make them unique are as follows [31]:

- Well-defined core-shell structure: Polymeric micelles with amphiphilic nature can accommodate water insoluble drugs and can maintain the stability within the living organism.
- Small size and larger surface area: Polymeric micelles with such small sizes (less than 100 nm) can travel through the smallest blood vessel (size around 200 nm), and the surface area of the micelle can be functionalized accordingly.
- Passive targeting: Blood vessels at the diseased site or tumor area become less permeable with a leaky vasculature compared to normal vessels, and polymer micelles, usually delivered by passive targeting, are accumulated at a malignant tumor site by their increased vascular permeability.

Drug loading into the polymer micelles from block copolymers is accomplished by dissolving the drug and polymer in the solvent and later diluting it with water. Finally, the solvent is dissolved to retain the particles [5]. Polymer micelles are administered intravenously, and targeting ligands may be attached to polymer micelles for site-specific drug delivery. The disadvantages of polymer micelles include lack of stable retention of drugs by micelles during

circulation, and drug molecules may diffuse from micelles and bind with proteins or cells before they reach the target site [31].

2.6.3 Dendrimers

A dendrimer is a macromolecule that is characterized by its 3D structure. Dendrimers are built with a starting atom like nitrogen to which carbon or other elements are attached by a repeated series of chemical reactions to form a spherical structure. Successive layers are added based on the application [16]. Dendrimers mainly consist of three parts: an initiator core; interior layers radically attached to the core, which is made with repeating units; and exterior layers attached to the outermost interior core [32], as show in Figure 8.

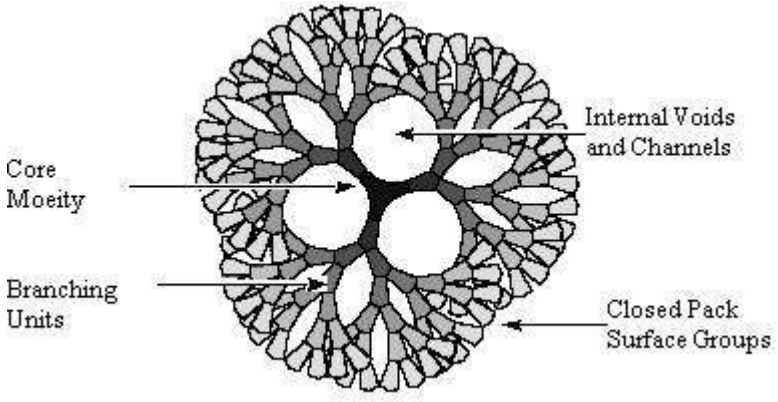


Figure 8: Dendritic structure [32].

As can be seen, dendrimers are formed by branching from the core to the exterior layers. Focal points are the branching points, which go from the core to the dendrimer surface. The number of focal points is counted to find the generation of the dendrimer, denoting the core part as zero. Certain physical parameters, such as size, shape, and surface chemistry, can be modified with the fabrication process of dendrimers. Drug delivery through dendrimers can be done by modifying its functional groups and then encapsulating the drug within the core or by attaching the drug to the surface groups of the dendrimer by a covalent bond or ionic interactions [32]. Dendrimers possess certain advantages that make it a good transporter for drug delivery

purposes. Some highly branched polymers or monomers have certain issues concerning lack of uniformity of functional groups, mono-dispersive nature, and even molecular weight distribution [33]. Dendrimers have exceptionally high drug loading efficiency, which allows the required amount of the dose to accumulate at the target site. Long circulation and slow release of the drug are offered by the dendrimer due to its increased molecular weight along with hydrodynamic volume when the drug is added to the dendrimer. Depending on the requirements, several dendrimers are available, such as for a targeted drug delivery, folate-conjugated dendrimers have a ligand attached. Dendrimers have some excellent properties such as a hydrophilic nature with high lipid solubility, and dendrimers like pH dendrimers, trigger the release of the drug based on pH at the target site [34].

Some experiments have been conducted to find the biocompatibility of dendrimers within the body, which revealed that they can be toxic to lungs. Polyamidoamine (PAMAM) dendrimers, also known as “artificial proteins,” have been of special interest for drug delivery purposes for their unique ethylenediamine core and repeated amidoamine branching. PAMAM dendrimers are the same size and shape as insulin, cytochrome C, and hemoglobin [35]. PAMAM dendrimers can be obtained as cationic or anionic. A cytotoxicity study was conducted by Li et al. by considering 12 dendrimers with different generations [35]. The experiment was conducted on human lung adenocarcinoma A549 cells by treating them with G1, G2, G3, G4, G5, G6, G7, G8, G3.5, G4.5, G5.5, and G7.5 generations, some of which were anionic and others cationic (G3, G4, G5, G6, G7 and G8). Cell death was observed with cationic dendrimers but not the anionic ones, so for analysis on cell death mechanisms, G3 dendrimers were chosen to represent the toxicity with cationic dendrimers, which were tested for caspase activity and DNA fragmentation, the two main symptoms for apoptosis. It was observed from the toxicity testing

that cell death was not due to apoptosis but was from an alternate pathway called autophagic cell death [35]. This process of autophagy by separating organelles and other proteins into a double-layer membrane vesicle called a autophagosome was studied, and it was found that excess autophagy can cause an independent cell death without following an apoptotic pathway [36].

2.6.4 Liposomes

Liposomes are highly convenient for drug-delivery purposes, and their surface properties can be modified based on a type of bilayer lipid, inclusion of covalent linkage of glycoprotein, or synthetic polymers [37]. Liposomes are spherical bilayer lipid vesicles between 50 nm and 1,000 nm in size. They have membranes usually in the form of phospholipids that have hydrophilic heads and hydrophobic tails. In the bilayers, the outer layers have heads that are attracted to the aqueous solution and the tails are within the layer, whereas the innermost layer has their heads attracted to the aqueous solution within the lipid. Drugs can be loaded into the aqueous solution within the lipid. Properties like biodegradable, less toxic and relatively less immunogenic makes liposomes more reliable for drug delivery purposes. Liposomes are fabricated based on the requirements of the intended use. For making the right fabrication choice, the following must be considered [38]:

- Physiochemical properties of the drug being entrapped along with the composition of the liposome, because some drugs are water insoluble, and therefore, liposomes are not a choice
- Type of aqueous solution in which the liposome needs to travel.
- Concentration and intensity of toxicity of the drug entrapped.
- Size of the liposome for the applications and its ability to disperse in the particular solution [38].

Liposomes can be classified based on their size, lamellarity, composition, and finally application [37]. Depending on size, liposomes can be classified as conventional and sterically stabilized. Conventional liposomes are made from neutral phospholipids with a small amount of cholesterol added to increase the stability in the plasma and a negative charge to prevent aggregation and also for efficiency. But liposomes are often treated as foreign elements, since they are recognized by the phagocytic system and cleared away by the reticulo endothelial system [39]. Stabilizing compounds are added to conventional liposomes to enhance their ability as well as reduce their reactivity. Adding ethylene glycol as a stabilizing agent increases the circulation time and prevents liposomes from being recognized as foreign material [40]. Based on the surface charge, liposomes can be anionic, cationic, and neutral. Based on lamellarity, liposomes can be unilamellar or multi-lamellar. Unilamellar liposomes have only one bilipid layer, and multi-lamellar liposomes have several bilipid layers. Liposomes are attached with surface ligands, which can be antibodies or ligands to deliver drugs to specific cells. Liposomes targeted with antibodies are called “immunoliposome.” When these liposomes are formed from sterically stabilized liposomes, they showed a better half life compared to those made with conventional liposomes [37].

Certain factors that affect the functioning of liposomes are size and surface charge. The uptake of conventional liposomes by RES was increased with an increase in the size of liposome. In sterilized static liposomes, slow uptake of the vesicle by the lung was observed at the size of 250 nm, and above 300 nm these liposomes were taken up by the spleen. Negatively charged and positively charged liposomes were cleared by RES more rapidly when compared to neutrally charged liposomes. Liposomes have poor drug-loading capacity, and not all drugs can be delivered due to the aqueous medium in the liposome [39].

2.7 Drug Targeting

Delivery of therapeutic agents to specific compartments within the cells is called targeted drug delivery. This technique results in high bioavailability of the drug at the target site. However, the targeted drug delivery aims to do the following [1]:

1. Target the drug specifically to cells or tissues.
2. Keep the drug out of non-targeted cells and organs.
3. Reduce the drug leakage during transport to the target site.
4. Protect the therapeutic agent from metabolism and premature clearance.
5. Release the drug at the target site for a desired period of time.
6. Be biocompatible, biodegradable, non-toxic, and non-antigenic.

Drug targeting can be classified six different ways [41]. The first classification is first-order targeting based on localization of the drug at the capillary bed of the organ (or) tissue. First-order targeting depends on the shape, size, and material properties of the carrier along with its routes of administration. If the drug is allowed to go through the selective passage within the target site (or) area, then it is considered second-order targeting. Third-order targeting is based on the drug being delivered within the cells by cell fusion, endocytosis or pinocytosis. Second-order and third-order targeting are dependent on the specific interaction between carrier, drug, and target cells. The second classification has organ, cellular, and subcellular targeting based on whether the drug is being transported to an organ, cells, or subcellular areas within the human body. The third classification depends whether the drug is being delivered naturally deposition or by forced delivery to the target site.

Figure 9 shows the natural distribution of a drug carrier through passive targeting [42]. The drug carrier travels through the MPS clearance, resulting in major accumulation in the liver

and spleen. After MPS clearance, the carrier with the therapeutic agents travels to liposomes, and the drug is released in the cell compartments. Passive targeting can also be done using the enhanced permeability and retention (EPR) effect to transfer the drug to the tumor site. This method is also called "selective targeting," because for this selective targeting, the size of the drug carrier system should exceed the normal size of endothelial fenestration (leaky vasculature caused due to tumor) to ensure that the carrier system crosses the inflamed endothelium and the circulation at the desired site. For these conditions to be satisfied the size of the drug carrier should not exceed 0.2 µm, and circulation time can be increased with stealth technology by increasing the surface hydrophilicity of the drug carrier. Active targeting requires that the drug carrier be guided to specific cells by attaching a homing device to the drug carrier for effective delivery or using magnetically responsive drug carriers. Thus, the active targeting carrier comprises a carrier, homing device, and drug. This homing device needs to be biodegradable and can be antibodies, carbohydrates, proteins, glycol lipids, vitamins, or other ligands.

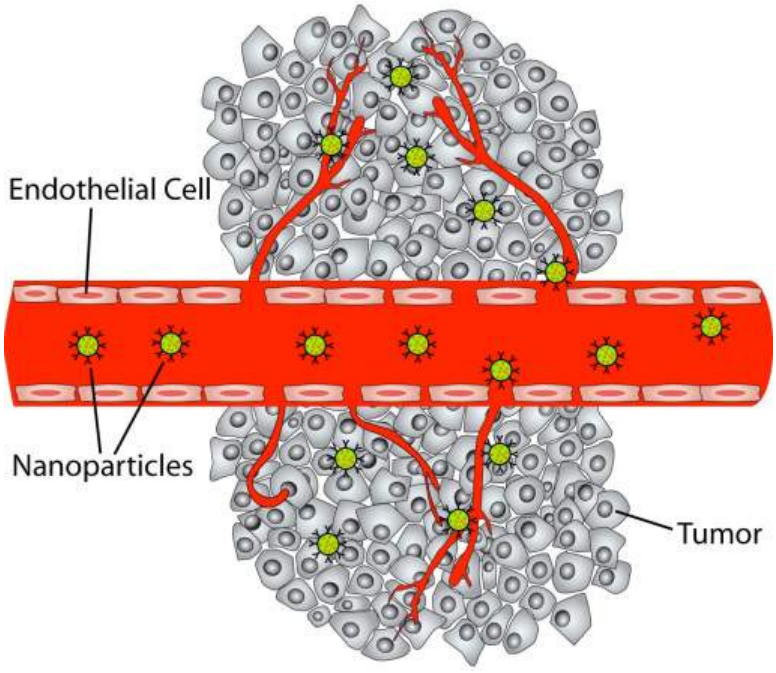


Figure 9: Passive targeting by nanoparticles in blood vessels [42].

The fourth classification is comprised of site-directed and site-avoidance targeting. Passive and active drug targeting can be considered site-directed targeting, since the drug is designed for the specific target site, but some specific sites should be avoided to prevent toxicity within the body. This is called site-avoidance targeting. The fifth classification is based on the transport of carriers across tissue at the target site. Biochemical targeting is the extravascular transport of a drug, with specific interaction between the target cell ligands and drug carriers [41]. Biomechanical transport of a drug occurs by transient opening of endothelial junctions due to osmotic imbalance etc. Biophysical targeting uses magnetic drag by a responsive drug carrier through the endothelium or uses a temperature-sensitive carrier for situations like hyperthermia in cancer treatment. Bioadhesive targeting combines the effects of biochemical and biophysical targeting. The sixth classification takes place after the drug reaches the target site. If the drug is released intracellularly, then it is considered carrier-dependent, and if the drug is released extracellularly, then it is considered carrier-independent.

2.7.1 Magnetic Drug Targeting

Magnetic drug targeting allows for the highest concentration of drug at the defined target, particularly away from the RES, using a magnetic field [43, 44]. The principle of magnetic drug targeting is that the intended drug and a suitable magnetically active component are combined into a stable formulation and injected into the body by way of the artery that supplies the target tissue. This is done in the presence of an external magnetic field with required field strength and gradient to retain the carrier to the target site [45]. New developments including the surface modifications of biodegradable magnetic polymer particles help in longer circulation times, which are helpful for drug delivery purposes [43, 46]. Magnetic drug targeting highly depends on the construction of strong magnets, which can produce high magnetic field gradients at the target

site. Most of the currently available magnets have inhomogeneous fields, which can only manipulate the particles against diffusion and blood stream velocities in a living system over a distance of a few centimeters from the sharp edge of an external magnetic pole [47, 44]. Two barriers associated with magnetic drug targeting are the following:

1. It is difficult to build sufficient field strength to focus on a small target area and to counteract the linear blood flows.
2. Circulation time depends reciprocally on particle size, whereas magnetic susceptibility is directly proportional to particle size.

Magnetic susceptibility is the ability of the applied field to magnetize a specific quantity of material. Among the appropriate materials, ferromagnetic materials have strong magnetic susceptibility and diamagnetic materials have low susceptibility [47, 48]. This susceptibility can be measured by molar weight, atomic weight, and volume of carrier. This measurement can be based on the magnetic ability of material per unit molecular weight, per unit atomic weight, and per unit volume of the carrier. The size and magnetic properties of the carriers must be optimized to reduce the unwanted uptake by RES and to prolong circulation time within the body.

2.7.2 Prodrug Approach

Prodrug can be described as an inactive form of a drug that needs to be activated upon reaching the specific target site. It actually occurs by covalent bonding between the drug and the chemical moiety, and was approached as one alternative to drug delivery. The prodrug concept works for site-specific delivery of drugs that are insoluble in an aqueous solution, toxic to normal cells and tissues, unstable, and incompletely absorbed across certain biological membranes like gastrointestinal mucosa and the blood-brain barrier. This concept is used to make drugs available at the target site by preventing any unwanted metabolism and to make the drug work with better

efficiency [49]. Prodrug should convert to an active form after reaching the target site. This reconversion can take place before absorption by the blood, after entering the blood stream, or after reaching the target site. This conversion into active form can be enzyme-mediated or pH-dependent. Figure 10 shows the basic prodrug approach, where the drug is attached to a molecule designated as the promoiety, in other words inactivation of the drug, which upon passing the biological barriers can be transformed through enzymatic or chemical transformation at the target site for the drug delivery [50].

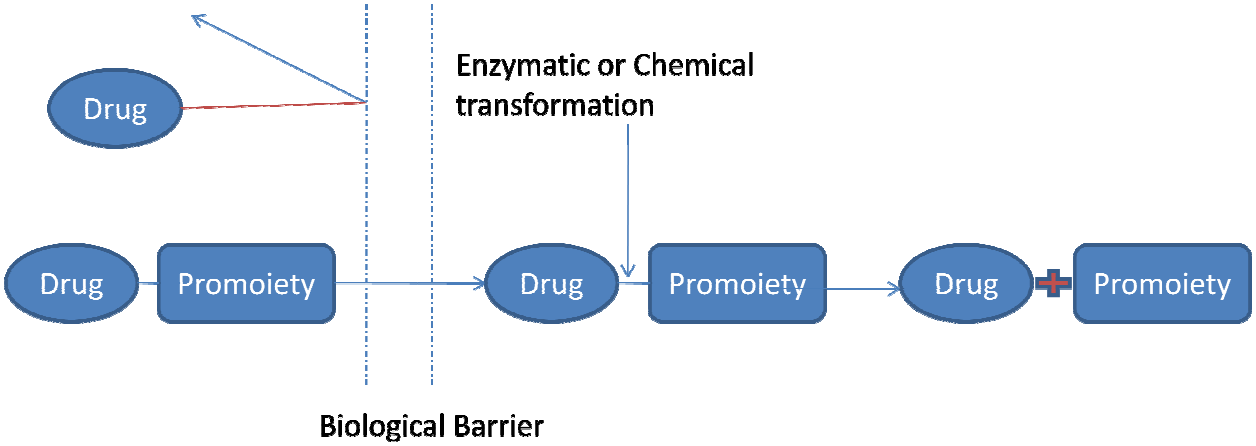


Figure 10: Prodrug approach [69].

The classical prodrug approach works as a cover to enclose some of the drug properties. To make the system work more efficiently, research was conducted on targeting the prodrug to a specific site, and it was proven by some authors that by optimizing certain factors, the prodrug approach can be used for targeted delivery as follows [51]:

- The prodrug must be transported and taken up by the target site based on the perfusion rate at the site.
- After reaching the target site, the prodrug must covert to an active component.
- The drug needs to be retained at the tissue for a desired period of time to complete the therapeutic process.

Prodrugs can be targeted by enzymes or by membrane transporters to the target site, Targeting the prodrug can be through “antibody-directed enzyme prodrug therapy (ADEPT)” or “gene-directed enzyme prodrug therapy (GDEPT)” [52]. These targeted prodrugs can even be conjugated with enzymes or polymers or ligands for the therapy. Certain requirements that the enzymes may possess to be suitable for ADEPT targeting are as follows [53]:

- Stable at 37°C
- Irreversible reaction
- Active near neutral pH
- High specificity
- Monomeric by choice
- Easily available

Selection of enzymes for targeting is a choice between microbial or mammalian. Microbial enzymes fulfill the requirement and are the best fit for targeting, but when introduced into mammalians such as humans, they can be recognized as foreign bodies, thus generating an immune response, which in turn causes toxic effects. Mammalian enzymes are compartmentalized and can accommodate the toxic drugs better and do not create any immune response when they enter the body. ADEPT enzymes act extracellularly, which means that the prodrug is activated outside the cell. After reaching the target site, the drug is converted to an active form by exploiting the fact that the target tissue contains the specific enzymes or high concentration of enzymes compared to the other normal cells. Targeting the prodrug can also be done by using membrane transporters, which use specified proteins carriers like amino acids, peptides, and glucose transporters. Similarly, GDEPT involves physical delivery of genes instead of enzymes to the target site, whereby after careful administration, the prodrug converts to an

active form after expression of an enzyme. The enzymes used in GDEPT act intracellularly and need cell-to-cell contact to maximize the killing of tumor cells [49]. Virus-directed enzyme prodrug therapy (VDEPT) is also a gene therapy using virus vectors for gene delivery and converts the harmless prodrug to an active and cytotoxic drug within the tumor cells with the help of enzyme transcription.

2.7.3 Routes of Administration

Drug delivery routes, otherwise called as routes of administration, can administer drugs absorbed by the body in different ways. The choice of routes can be based on the type of disease, organs affected by the disease, the effect desired, or the product available. The main routes of administration can be divided into the following [1]:

- Enteral routes: Enteral routes include drug delivery through oral or rectal pathways. In these routes, the drugs can be taken directly using self control for systematic effects at variable concentrations. Due to the high acid concentrations and presence of digestive enzymes in the gastrointestinal (GI) tract, this can cause degradation of the drug before reaching the site of absorption or before it enters the blood stream, and the lower pH in the digestive system may result in transition of the drug to an insoluble form, which reduces the drug's bioavailability at the target site. Some drugs even cause irritation in the GI tract and cannot deliver the drug to the target site. Despite these disadvantages, this route has the highest patient convenience with a moderate dosage control along with ease in taking the medicine [13].
- Parenteral routes: Parenteral routes introduce substances into the body other than through the GI tract. All parenteral routes are shown in Figure 11. The drug's administration can be intravenous, intramuscular, intra-arterial, and subcutaneous. Drugs by these routes are

usually injected by syringes, which are non reusable with autodestruct or with retractable needles or the ones that lock after injection. All parenteral routes have rapid onset of action, high bioavailability at the site of action, avoid drug degradation or premature clearance at the GI tract, and are convenient for comatose patients and those who cannot digest orally. Drawbacks include painful injection and limitation with the delivery of protein products [1].

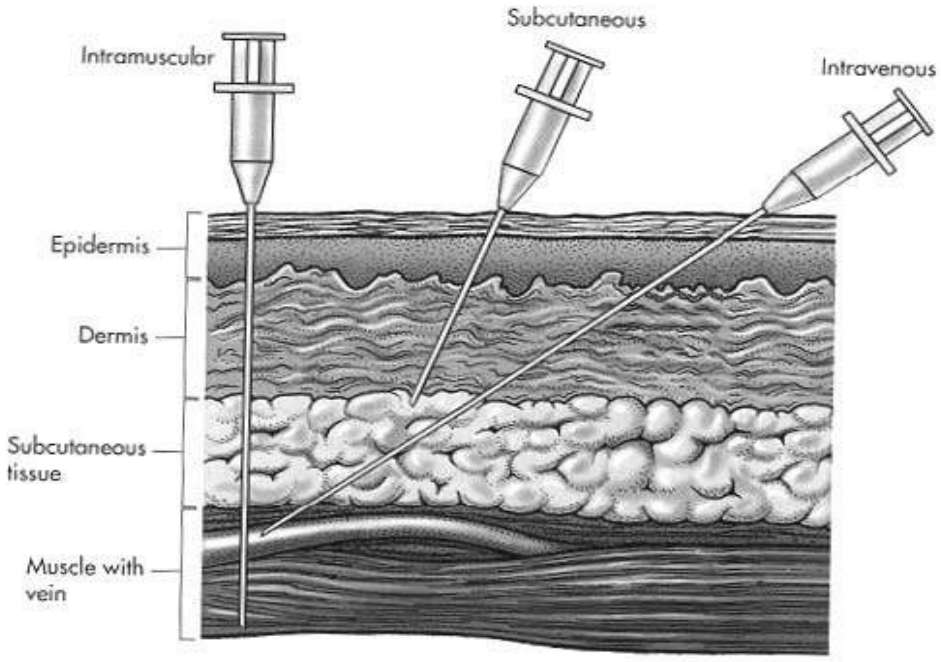


Figure 11: Parenteral routes [54].

- Subcutaneous routes introduce the drug to subcutaneous fatty tissue using a hypodermic needle. Certain factors that influence drug delivery include the size of molecules. Larger molecules have lower penetrations compared to smaller molecules. Also, vascularity and amount of fatty tissue affect absorption of the drug into the body. Subcutaneous routes usually have slower absorption rates and low onset of action compared to intramuscular and intravenous injections. Disadvantages include local complications like irritation and

pain at the site of injection, and having to change the injection site to avoid accumulation of an unabsorbed drug, which may cause tissue damage [1].

- Intramuscular injections involve the introduction of the drug deep into the deltoid or gluteal muscles. Here the onset action is faster than subcutaneous routes but slower than intravenous injections. The rate of absorption depends on the physiochemical properties of the drug, the blood circulation, and the state of muscle activity. Disadvantages include pain at the injection site, limitation of injected amount according to muscle mass, and degradation of peptides at the drug injection site. Intramuscularly, the drug action is slower with long-lasting effects at peak drug concentrations from one to two hours. Factors that affect release of the drug are concentration, particle size, physical form, and volume of the drug.
- Intravenous injections involve injecting the drug into a superficial vein using a needle or a catheter. This has rapid onset action, and the rate of infusion can be controlled for prolonged and continuous administration. Particle size should be between 0.1 μm and 7 μm in order to be taken up by the liver and spleen. Disadvantages include infections due to catheter introduction and immune reactions.

Other routes of administration are transdermal, nasal, transmucosal, colorectal, and pulmonary [1].

2.8 Cell Death Mechanisms Due to Nanoparticles

The use of nanotechnology for health care requires a combination of many disciplines, including physics, chemistry, chemical and mechanical engineering, and clinical medicine. The design of a hip implant carried out at the nanolevel mimics the mechanical properties of human bone, thereby preventing stress-shielding and loss of surrounding bone tissue. Metal oxides

transition metals being used for drug delivery purposes and information about this toxicity are of vital importance [39]. Materials used in medicine must be biocompatible, non-toxic, non-carcinogenic, non-mutagenic, and non-antigenic. Particle size and surface area are two important aspects from a toxicological perspective [14].

As particle size decreases, it accommodates more atoms or molecules on its surface, and changes that occur in the physiochemical and structural properties due to the decrease in size can be responsible for numerous material interactions [26], which may lead to toxic effects. Inducing these solid materials that can penetrate through cells initiates a process called foreign body transformation, which is caused as the result of mechanical or oxidoreduction damage to chromosomes or the nucleus [55]. Shrinkage in size can even cause discontinuous crystal planes, which increases the number of surface defects along with disruption of well-structured electronic configuration of the material, thus leading to the alteration of electronic properties of the nanoparticles. When these materials enter the body, they tend to establish surface groups, which can sometimes act as reactive sites [28]. A study of different nanoparticles showed different adverse effects on the body; for example, carbon black nanoparticles cause oxidative injury, inflammation, and cytotoxicity, whereas titanium oxide particles cause DNA damage [55]. Upon uptake, nanoparticles can enter various cell parts, such as the outer membrane, cytoplasm, and mitochondria, as well as the nuclear membrane or nucleus. Therefore, depending on their localization, nanoparticles can even damage DNA [56]. Nanoparticles are internalized into endothelial cells, pulmonary epithelium, red blood cells, platelets, and nerve cells based on the size of particles.

The nanoparticle mechanism of inducing pro-inflammatory effects is unknown, but it does cause oxidative stresses that result in cell death. Oxidative stress can be caused by the following sources [7]:

1. Reactive oxygen species (ROS): ROS can be defined as reactive molecules with oxygen atoms that are released from nanoparticles with both oxidants and free radicals on the surface. ROS cause oxidative stress and has even proven to damage cells by altering proteins and disrupting DNA by interfering with signaling functions, etc [7].
2. Transition metals: Nanoparticles formed from transition metals like iron and copper can release ROS by acting as a catalyst in Fenon-type reactions. For example, as shown in the equation below, reduction of hydrogen peroxide (H_2O_2) with ferrous iron (Fe^{2+}) results in a highly reactive hydroxyl radical (ȮH)

$$\cdot O_2^- + H_2O_2 \xrightarrow[Fe]{} \cdot OH + OH^- + O_2$$

3. When nanoparticles come in contact with mitochondria, they affect the functioning of the cell, thus resulting in physical damage and thereby contributing to oxidative stresses.
4. Upon phagocytosis of nanoparticles, alveolar macrophages and neutrophils are released, and this leads to the generation of ROS. Alveolar macrophages even initiate inflammation in the lungs [7, 57].

The increase in free radicals and the release of ROS occurs more in nanoparticles than in bulk materials due to their high surface area [58]. Oxidative stress also causes lysosomal membrane permeabilization (LMP) by oxidizing the lipid membrane and resulting in destabilization of the lysosomal membrane. LMP alters the functions of the lysosomal membrane, causing release of cathepsins and other hydrolases from the lysosomal lumen to the

cell. This causes cell death which can be caspase-dependent apoptosis, caspase-independent apoptosis, or even necrosis, followed by high levels of LMP [58].

Inflammation is a general biological response of the body to any injury or infection by pathogens. Inflammation helps body with regeneration of healthy tissue, but excess inflammation causes diseases, several experiments conducted on nanoparticles induced inflammation proved that exposure to nanoparticles causes inflammation with the intensity of inflammation based on size and chemical composition of nanoparticles [8]. Oxidative stress due to nanoparticles triggers the release of pro-inflammatory mediators or cytokines resulting in cell death. Cell death can also be caused by damage to mitochondria without any inflammation. Antioxidants are produced within the body to remove the oxidative stresses, but increase in the generation of ROS sometimes overcomes the antioxidant defense system resulting in oxidation followed by destruction of cellular biomolecules like DNA [7, 57].

Cell death due to nanoparticles is mostly through Apoptosis also called as programmed cell death or cell suicide. During apoptosis cells degrade by them self resulting in cell shrinkage and there by disintegrate into small apoptotic bodies to remove unwanted or dysfunctional cells. Apoptosis is a controlled process where the cell will be phagocytosed by macrophage before letting the cell contents to the neighborhood [56]. Apoptosis can be triggered through intrinsic or extrinsic pathways. Extrinsic pathway is through ligands from other cells, these ligands bind to the transmembrane death receptors to initiate the apoptotic process. In the intrinsic pathway the cell death is mainly due to cellular stress specifically by mitochondria stress caused by DNA damage [59]. Figure 12 explains the apoptosis process, where upon cell death, the cell shrinks and the nucleus collapses disintegrating into small bodies which get cleared away as a waste [59].

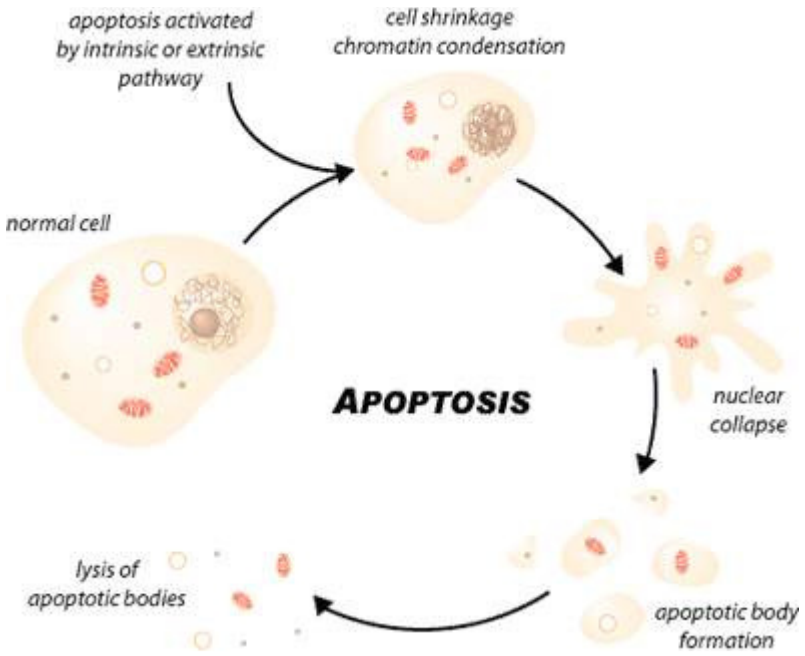


Figure 12: Apoptosis—programmed cell death [59].

Necrosis is other kind of cell death. Necrosis is mostly due to external factors such as infection, poison, toxins etc. Cells that die due to necrosis don't send any signals to the immune system which makes it hard for the nearby phagocytes from locating the dead cells. These dead cells burst out the content to the surrounding tissues, spreading the dead cells and debris in the nearby site [60].

Toxicity of nanoparticles is dependent on certain physical parameters like dose, mass, number, composition, aggregation etc. lower doses of small nanoparticles are found to be more toxic than larger particles with higher dose, this was observed as toxicity is dependent on surface area of the particles small particles have higher surface area compared to larger particles which explains that toxicity increases with decrease in size of the particles [8]. Concentration of nanoparticles shows it impact as it depends on type of nanoparticles and its aggregation tendency, because nanoparticles made of cobalt ferrite are observed to aggregate and due to this aggregation it makes their size larger to be cleared away by macrophages or as they tend to

behave like larger particles, it reduces toxicity, so concentration of nanoparticles increases the aggregation tendency their by reducing the toxicity. Finally chemistry of the nanoparticles is also important as it varies with the crystalline structure of the particle can change when nanoparticles come in contact with the solution even though they are made of same composition. All these issues need to be taken care off before synthesis of nanoparticles and requirement.

CHAPTER 3

EXPERIMENTAL PROCEDURE

Cobalt Ferrite ($CoFe_2O_4$) and Nickel Ferrite ($NiFe_2O_4$) nanoparticles are synthesized using facile sol-gel method. These magnetic nanoparticles are combined with PLGA (poly lactic co- glycolic acid) to fabricate nanonomposite spheres at different percentages of the inclusions. Below is the general description of our experimental procedure.

3.1 Sol-Gel Method

Nanocrystalline powder of cobalt ferrite and nickel ferrite can be prepared by many processes namely Auto-Combustion, Co-precipitation, Citrate gel, Micro-emulsion, and Hydrothermal process [61]. Sol-gel process is widely used among the process available like co-precipitation, and hydrothermal method for its simple process, impurity free and ultra fine particles. Cobalt ferrite and nickel ferrite nano-powder were synthesized by a facile sol-gel method based on polyesterification reaction between citric acid, benzoic acid and ethylene glycol .The sol-gel method used in this thesis mainly consists of three steps, starting with complexation between metal ions and citric acid and using iron citrate instead of iron nitrate accelerated the complexation process [62] ,when ethylene glycol is added to the solution polymerization reaction takes place with a polyester network. Benzoic acid acts as a secondary reagent and accelerates the polymerization and gel formation [61]. Materials used to synthesize Cobalt ferrite ($CoFe_2O_4$) include cobalt nitrate (Co $(NO_3)_2 6H_2O$), Iron citrate ($FeC_6H_7O_7$), Benzoic acid (C_6H_5COOH), Ethylene glycol ($(CH_2OH)_2$) purchased from Sigma Aldrich and Citric acid ($61C_6H_8O_7$) purchased from Fisher Scientific. A molar ratio of 1:4 is maintained between the metal nitrates and citric acid. Cobalt nitrate hexahydrate and Citric acid are used as starting materials; they are mixed with 50 ml of distilled water and placed on hot plate with a stir bar for continuous stirring

at a temperature of 30-40°C. As shown in Figure 13, the apparatus is set under the fume hood and the Sol Gel method is carried without any pH adjustment and aging of the solution. Iron citrate is added to the solution and the temperature is set to 75°C stirring at 800 rpm. After the solution turns to bright brown color, required amount of Ethylene glycol and Benzoic acid are added to the solution and made to stir continuously till the solution turns to dark brownish gel. This viscous gel is placed in oven at 150°C for 24 hours, this treatment turns the gel into powder which is taken out of oven and crushed to fine powder and again placed in oven at 750°C for annealing purposes for about one hour by a heating rate 10°C/min which can be observed from Figure 14. The same method is used to prepare the nanocrystalline powders of Nickel ferrite using Nickel nitrate hexahydrate ($Ni(NO_3)_2$) and Citric acid ($61C_6H_8O_7$) as starting materials.

Figure 13: Preparation of cobalt ferrite using sol-gel process.

Figure 14: Drying and annealing the powdered cobalt ferrite.

3.2 Magnetic Nanocomposite spheres fabrication

Figure 15 explains the process of nanomagnetic polyspheres fabrication using the oil-in-oil emulsion/solvent evaporation technique. The PLGA (poly lactic co-glycolic acid) purchased from Sigma Aldrich is used as polymer to bind the magnetic nanoparticles to form magnetic nanocomposite spheres. Cobalt ferrite and nickel ferrite nanoparticles are added at different percentages to the total weight of the batch to test the toxicity of the metal ferrites. Acetonitrile (C_2H_3N), purchased from Acros is used as polymer solvent and, Paraffin oil from Sigma Aldrich is used in the second phase preparation combined with Span 80 (Fluka) which acts as a surfactant, Petroleum ether and Carbon tetrachloride (CCl_4) from Sigma Aldrich for oil removal and then N-hexane (C_6H_{14}) for washing polyspheres from any dirt. Finally, a membrane filter is used at the end to separate the particles from the solution/dispersion.

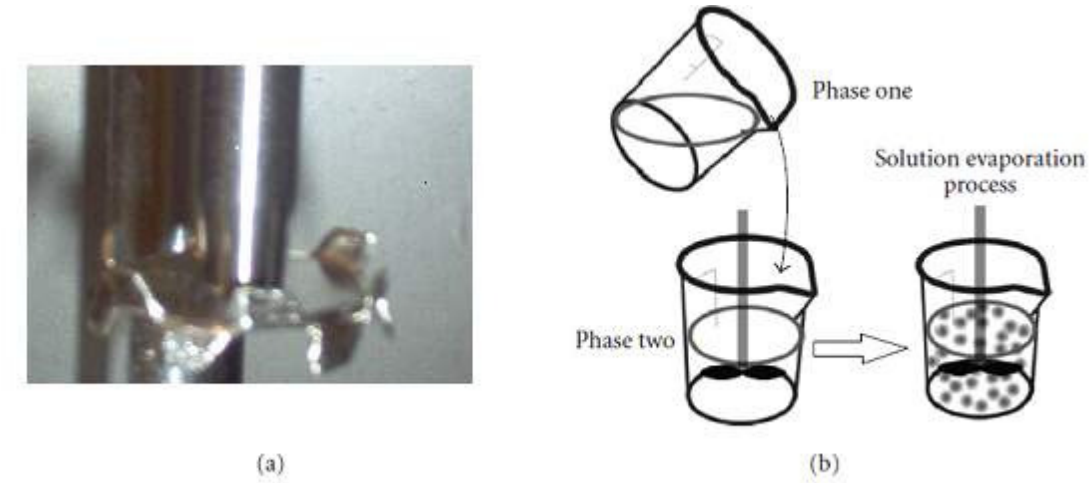


Figure 15: (a) Specially designed blades for phase-II oil-in-oil emulsion/evaporation technique, (b) Oil-in-oil emulsion/evaporation procedure [51].

This oil-in-oil emulsion/solvent evaporation technique consists of two phases including aqueous phase and oil phase. The total weight of the batch is maintained to be 0.0625g for all the samples but the weight of the cobalt ferrite and nickel ferrite were varied. The weights of polymer and the nanoparticles are given in Table 1

TABLE 1

WEIGHT OF PLGA, COBALT FERRITE AND NICKEL FERRITE FOR DIFFERENT SAMPLES

SAMPLE	PLGA CONCENTRATION (g)	MAGNETIC NANOPARTICLE CONCENTRATION (g)
$CoFe_2O_4$ (5%)	0.0594	0.0031
$CoFe_2O_4$ (10%)	0.0563	0.0063
$CoFe_2O_4$ (15%)	0.0531	0.0094
$NiFe_2O_4$ (5%)	0.0594	0.0031
$NiFe_2O_4$ (10%)	0.0563	0.0063
$NiFe_2O_4$ (15%)	0.0531	0.0094

Figure 16: Phase I: Mixing PLGA in acetonitrile.

As shown in Figure 16, the required amount of PLGA is crushed in a Petri dish and then added to 5ml of Acetonitrile in a flask and placed on mixer with a stir bar and stopper on the flask to prevent Acetonitrile from evaporating. After dissolving PLGA completely the stir bar is taken out and the magnetic particles are added in specified ratios, then the flask is placed on a sonicator for dispersing the nanoparticles. This action completes the first phase [63, 64].

Figure 17: Adding phase-I to phase-II

40 ml of Paraffin oil is then added to 0.2ml of Span 80 in a separate beaker. This mixer is placed under mixer operating at 7000 rpm. First phase is added to the mixture drop wise using a burette to separate the particles from agglomeration and clustering which can be seen in Figure 17. The mixer is allowed to run for one hour and 30 minutes. The mixture is poured into centrifuge tubes to spin at 17000 rpm for 20 minutes, these spheres tend settle in the bottom of the tube at the end of operation. The tubes with the spheres attached in the bottom are filled with N-hexane and Petroleum ether for oil removal in 20:1 ratio, for example, for every 100 ml of N-hexanes 5ml of Petroleum ether is added. The tubes are again placed in the centrifuge for 20 minutes at the same speed, and then .the process is repeated with N-hexanes twice to clean the particles for any remains of oil and surfactant. After centrifuging, the tubes are refilled with N-hexane and 0.05% Carbon tetrachloride for extra purification and then placed in the sonicator for 10 minutes to disperse the particles completely. 200 nm pore size membrane filter is used to filter the particles with the help of vacuum pump and the particles are collected in the end. Figure 16 shows the obtained nanocomposite spheres from filtration process. There were some losses during the fabrication process at certain steps:

- While transferring the first phase to second phase under mixer using burette. Some particles were observed to be deposited at the edges and at inclined areas in the burette,
- After completing the second phase and transferring the contents from breaker to centrifuge tubes due to the viscosity of the oil, some particles were settled at the bottom.
- While emptying the tubes to membrane filter under vacuum and
- While scratching the nanospheres attached to the filter after filtration. Due to the above losses the productivity was observed to be from 40 – 60%.

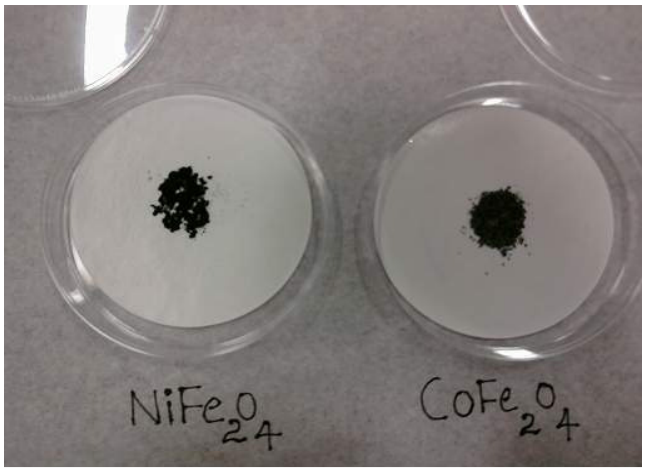


Figure 18: Nanocomposite spheres made from cobalt ferrite and nickel ferrite

3.3. Cytotoxicity Test Method

In this report, the toxicity caused by the magnetic nanocomposite spheres fabricated in the laboratory is tested by cell growth using a colorimetric method which measures the color to find the cell viability after exposing the cells to the samples at different concentrations for a limited period of time. Measuring the cell viability and growth has been a valuable tool to find the drug sensitivity, cytotoxicity, response to growth factors and cell cultivation [65]. Cell proliferation is affected by growth factors that bind to surface receptors, connecting to signaling molecules. These molecules trigger the transcription of DNA that regulates the production of proteins necessary for the cell division. Dysfunction at any one of these steps effects cell growth that gives rise to pathological symptoms effecting human health [56]. These cell proliferation assays are responsible for understanding the mechanisms that alter or regulate the cell cycle like growth factors and drugs, etc [68].

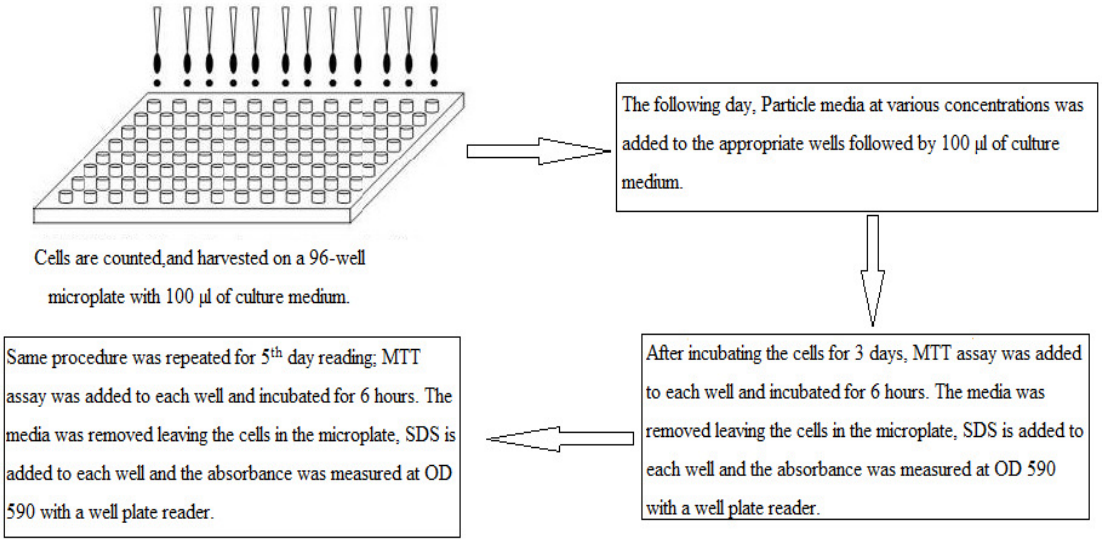


Figure 19: Cytotoxicity Test procedure [55].

Figure 19 portrays the procedure of the cytotoxicity test conducted in our nanotechnology lab. This test was conducted using MTT assay. MTT is a standard laboratory test which measures the cell proliferation and cytotoxicity using colorimetric method. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt when incubated for 4-6 hours, reacts with the cell mitochondria and produces a purple or blue colored formazan product. This formazan product upon adding a detergent which is SDS (Sodium Dodecyl Sulphate) breaks the cells to bring the formazan out which leaves a blue color in the microplate and thereby measured for cell viability based on the color in the microplate using a well plate reader at OD 590 [65].

Cytotoxicity test for nanocomposite spheres made of cobalt ferrite and nickel ferrite was tested using raw cells which are mouse microphage were used as a model cells and the test was conducted using a calorimetric method with MTT assay. The dilution ratio used was 1:5. Cells are counted and harvested on a U-bottomed 96-well microplate with a 100 µl of culture medium and incubated for 24 hours at 37°C for the cells to get seated within the plate. After 24 hours of

cell seeding, 500 µg of the sample is added to the first well in the each row, the concentration of the sample added is reduced by half to the next well in the same row, to find the toxicity at different concentrations for the sample with same composition. Then 100 µl of culture medium is added to each well and then placed in the incubator at 37°C for the 3rd day readings. The figure below (Figure 20) shows the microplate after adding the nanoparticles at their respective concentrations to each well and the concentrations of sample in each well are tabulated below in Table 2.

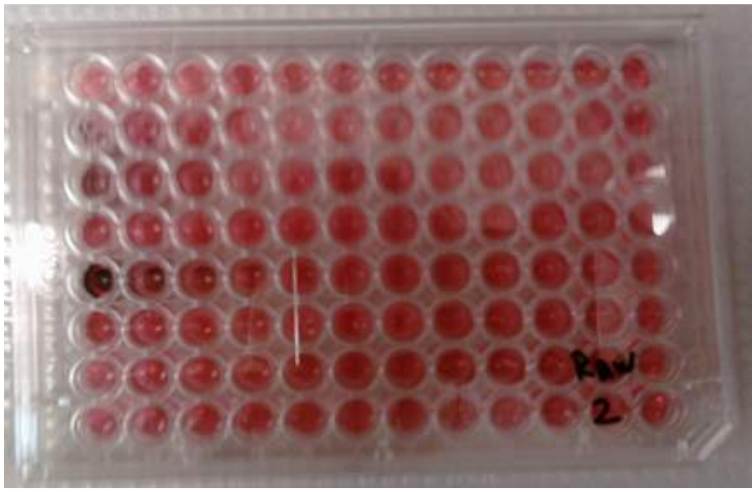


Figure 20: 96-well microplate with sample at different concentrations in each well.

TABLE 2

SAMPLE CONCENTRATIONS (µg/ml)

		1	2	3	4	5	6	7	8	9	10	11	12
1	A	500	250	125	62.5	31.3	15.6	7.8	3.9	2	1	0.5	0.24
2	B	500	250	125	62.5	31.3	15.6	7.8	3.9	2	1	0.5	0.24
3	C	500	250	125	62.5	31.3	15.6	7.8	3.9	2	1	0.5	0.24
5	D	500	250	125	62.5	31.3	15.6	7.8	3.9	2	1	0.5	0.24
6	E	500	250	125	62.5	31.3	15.6	7.8	3.9	2	1	0.5	0.24
7	F	500	250	125	62.5	31.3	15.6	7.8	3.9	2	1	0.5	0.24
8	G	500	250	125	62.5	31.3	15.6	7.8	3.9	2	1	0.5	0.24
Medium	H	0	0	0	0	0	0	0	0	0	0	0	0

After 3 days in incubator, it was observed from the picture taken (Figure 21) that the wells with less sample concentration has more color which indicates that more cells were alive.MTT assay diluted at a rate of 5mg/ml is added the plate at 20 µl to each well and incubated for 6 hours after which the culture medium and MTT are taken out from the well leaving the cells attached to the bottom of the plate. Figure 22 shows the microplate after incubating for 6 hours for the insoluble formazan to form within the cell. The wells are then filled with 100 µl of SDS and placed in the incubator again for the next day reading. Figure 23 shows the clear color variation that says most of the cells exposed to higher concentration died and reducing the concentration have increased the cell viability. The contents in the microplate are transferred to another microplate before measuring the absorbance in the Elisa reader at OD 590. Transferring is done to get the correct reading as the sample particles might add the color in the well which can be read by reader as formazan. Care must be taken that there are no particles and air bubbles when the microplate is being feed to the reader. Same procedure is repeated for the 5^{th} day reading, where the cell are cultured and incubated at 37°C and with 95% air and 5% CO_2 for 5 days and on 5^{th} day MTT is added to the wells and incubated under the same conditions for 6 hours to reduce the MTT to formazan and then treated with SDS to break the cells and the absorbance was observed at OD 590 in the Elisa reader.

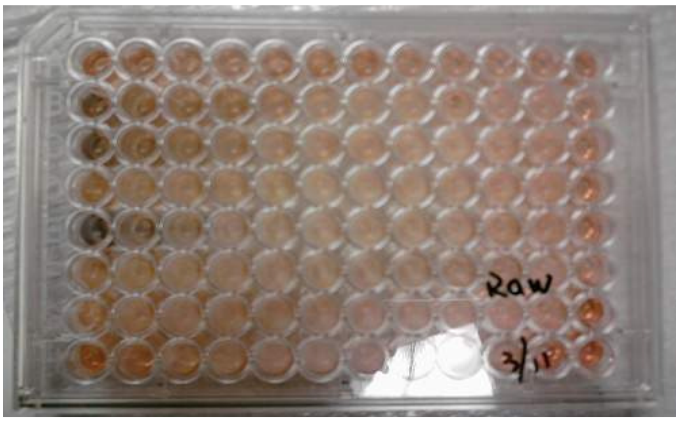


Figure 21: After 3 days in Incubator before adding MTT

MTT was being widely used for neural toxicity test with preclinical drug studies and also to find the cell viability [66]. The MTT assay is used for cells that grow exponentially and for non-radioactive sample testing; Variety of compounds can be tested with this assay but the compounds that being tested are expected to be soluble in the culture medium. This method is semi-automated, simple, inexpensive, can be applied for many compounds and very sensitive which means it can detect with fewer number of cells. MTT is a tetrazolium salt which is a heterocyclic organic compound that forms insoluble formazan on reduction within the cells.MTT was observed to be reduced by reaction with mitochondria and forms a Granular formazan. These formazan was found in the endosomal/lysomal compartment within the cell. MTT enters into the cell by endocytosis and reduces to formazan in a granular form. The reduction of MTT to the formazan crystals is time dependent, with increase in the incubation time; these formazan crystals appear bigger, darker and closer to the plasma membrane [66].The capability of the cells to reduce the MTT signify the mitochondrial activity, thus can be measured as the cell viability.

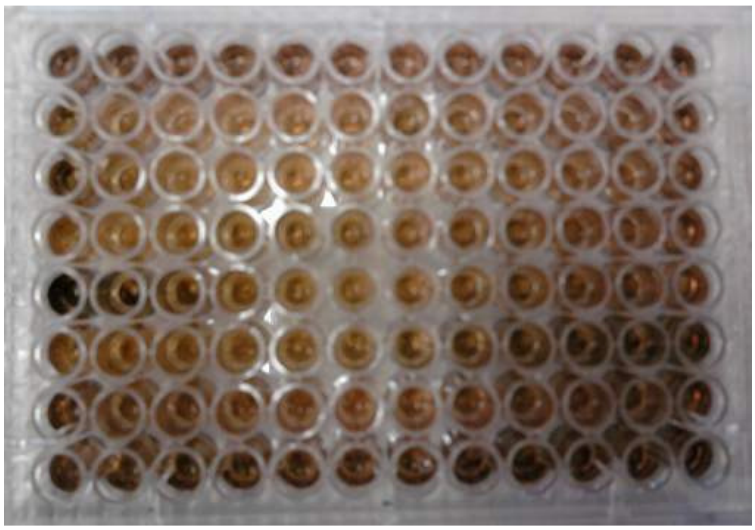


Figure 22: Microplate after adding MTT and incubation for 6 hours.

MTT reduction by cells was observed to be influenced by certain factors. MTT reduction depends on the cell line. Different cell lines have different reduction percentages and also the D-

glucose concentration in the culture medium increased the reduction, MTT reduction reduced with the depletion of D-glucose in the culture medium [67]. Experiments conducted proved that renal carcinoma cells have depleted 90% of the glucose in 4 days, where as other cell lines from the CNS, that grow slowly have depleted only 15% during the same time interval and this decrease in D-glucose have directly affected the reduction of MTT within the renal carcinoma cells , but the reduction rate was constant in CNS cell lines ,which says that MTT reduction depends on the cell lines. Some more experiments were conducted to find the influence of D-glucose on the MTT reduction, Leukemia cells were observed for specific activity with MTT in a D-glucose rich medium as well as glucose free medium, it was found that the when incubated with 5% CO_2, D-glucose concentration medium is related to atmospheric CO_2 in the incubation. So when incubated with 5% CO_2, the reduction activity was good in glucose rich medium, than the glucose free medium [67].

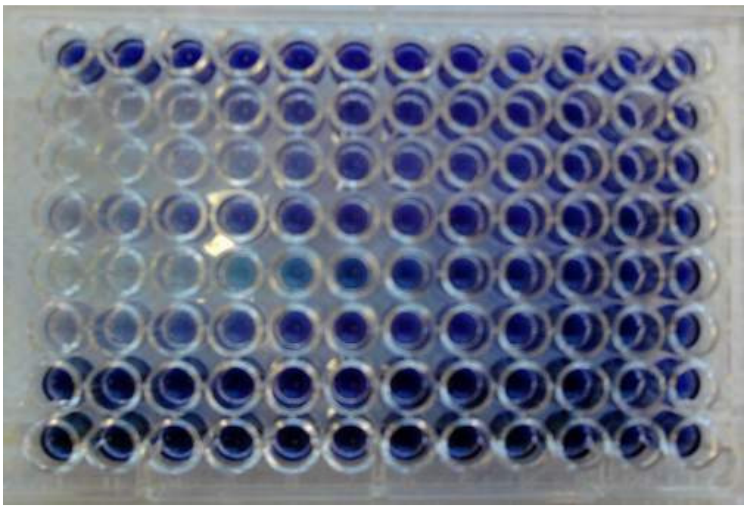


Figure 23: Microplate with SDS

This influence of carbohydrate was because some cell lines can metabolize the CO_2 quickly compared to others. The MTT reduction to its corresponding insoluble formazan was directly proportional to the incubation time, the formazan was observed to be nearer to the plasma membrane with increase in the incubation time. The reproducibility of the MTT assay is

very poor, the same microplate cannot be used for the other day reading and is also time dependent process.XTT and Alamar blue are other to assays mostly used for cell viability.XTT forms a soluble formazan which simplifies the process and the plate with Alamar blue assay can be read for different day reading i.e., the same plate can be used again.

CHAPTER 4

RESULTS AND DISCUSSION

The aim of this study was to establish the toxicity of the selected magnetic nanocomposite spheres to mouse microphage as well as to investigate the effect of nanoparticles concentration on the cytotoxicity. To read the microplate, Soft Max software 5.2 was used and the readings are tabulated from the 3rd day and 5th day readings respectively: Refer to Tables 3 and 4.

TABLE 3

RESULTS FROM THE THIRD-DAY READINGS

Medium	2.06	2.11	2.08	2.13	2.15	2.00	2.11	2.24	2.31	2.33	2.31	2.11
5% Co-3rd	0.09	0.15	0.16	0.13	0.20	0.34	0.80	1.04	1.35	1.55	1.84	1.90
5% Ni-3rd	0.49	0.63	0.31	0.20	0.28	0.53	0.92	1.21	1.48	1.85	2.00	2.12
10% Co-3rd	0.07	0.09	0.09	0.11	0.13	0.24	0.32	0.25	0.40	0.44	0.65	0.87
10% Ni-3rd	0.07	0.08	0.08	0.14	0.17	0.27	0.42	0.53	0.76	0.81	0.93	1.15
15% Co-3rd	0.03	0.04	0.05	0.05	0.07	0.11	0.15	0.27	0.28	0.40	0.51	0.70
15% Ni-3rd	0.06	0.05	0.06	0.07	0.10	0.18	0.66	0.52	0.59	0.64	0.78	0.96

TABLE 4

RESULTS FROM FIFTH-DAY READINGS

Medium	2.16	2.44	2.44	2.57	2.49	2.54	2.69	2.61	2.76	2.64	2.79	2.29
5% Co-5th	0.21	0.30	0.20	0.47	0.56	0.87	0.98	1.10	1.21	1.49	2.08	1.96
5% Ni-5th	0.27	0.46	0.21	0.26	0.61	1.14	1.23	1.19	1.50	1.67	2.15	2.11
10% Co-5th	0.15	0.15	0.26	0.18	0.55	0.78	0.80	1.08	1.00	1.20	1.26	1.81
10% Ni-5th	0.22	0.25	0.39	0.58	0.66	0.92	0.95	1.07	1.12	1.59	1.23	1.96
15% Co-5th	0.05	0.05	0.09	0.09	0.11	0.18	0.14	0.60	1.38	1.45	1.70	1.91
15% Ni-5th	0.05	0.07	0.11	0.12	0.26	0.25	0.23	0.90	1.64	1.73	1.81	1.91

From these results some conclusion can be made by plotting the graphs to compare various samples with different concentrations of magnetic nanoparticles. After conducting a literature review on these magnetic nanoparticles and their interactions with the cells we believe that they can be toxic to the raw cells based on the concentration they are exposed. So for these reasons cytotoxicity analysis was conducted to find the cell proliferation rate, when the cells are cultured and were exposed to certain amount of sample concentration.

4.1 Cytotoxicity of PLGA spheres

PLGA is a biodegradable polymer and is being used in biomedical applications from 1970 [12].nanocomposite spheres fabricated from PLGA are tested for cytotoxicity to observe whether the fabrication process used i.e., oil in oil evaporation/emulsion technique does result in any residues that can effect when spheres come in contact with the cells. Theoretical assumption is the cells exposed to PLGA spheres should have same cell growth when compared with culture medium but, experimental results showed the difference in cell viability which might be due to the residues from the fabrication process like paraffin oil or carbon tetra chloride etc. Results from Table 5 are plotted in graph 24 and 25 based on 3rd day and 5th day readings.

TABLE 5

CELL VIABILITY WITH PLGA SPHERES AND CULTURE MEDIUM

	1	2	3	4	5	6	7	8	9	10	11	12
Medium-3rd	1.81	1.83	1.79	1.70	1.66	1.65	1.62	1.78	1.78	1.76	1.89	1.82
PLGA-3rd	1.23	0.82	0.79	0.97	1.35	1.08	1.48	1.39	1.53	1.46	1.59	1.35
Medium-5th	1.94	1.94	1.92	1.74	1.67	1.66	1.62	1.74	1.93	1.93	2.17	2.13
PLGA-5th	1.21	0.89	0.83	0.89	1.07	1.41	1.64	1.73	1.76	1.89	1.86	1.71

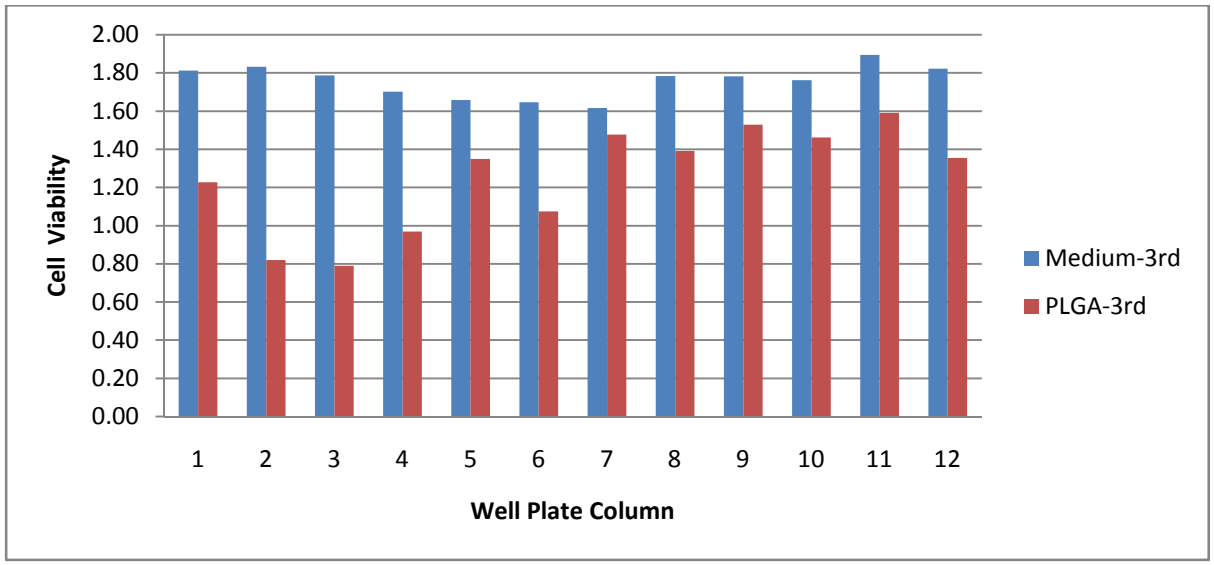


Figure 24: Cell Viability with PLGA from 3rd day readings.

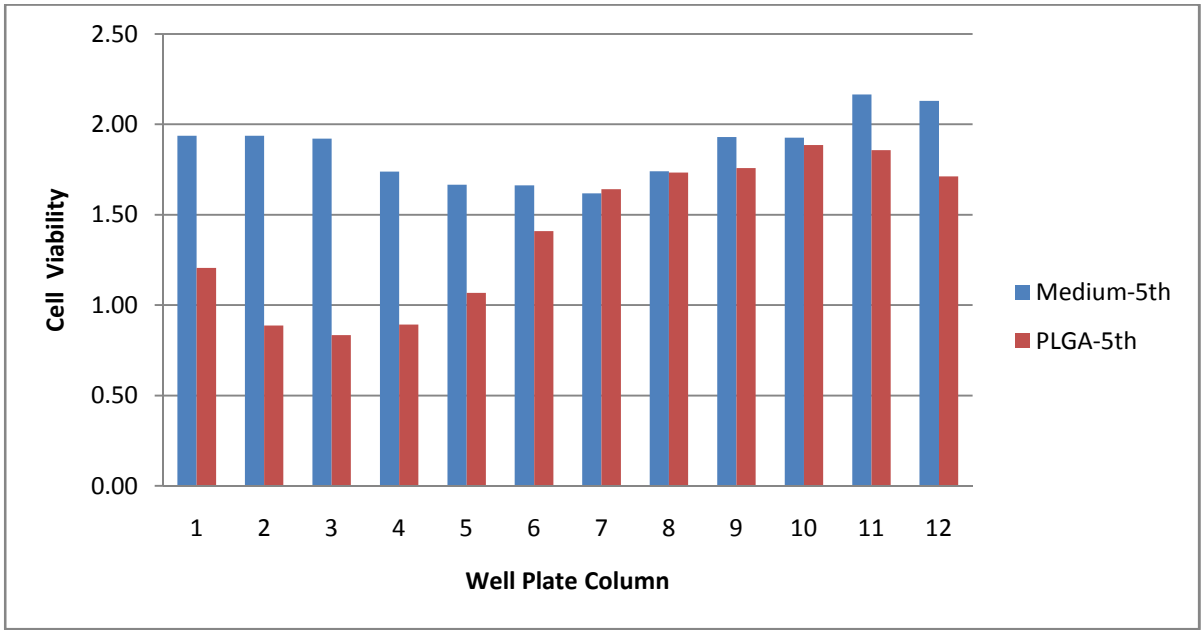


Figure 25: Cell Viability with PLGA from 5th day readings.

It can be observed from the graphs that the first well which has the highest particle concentration has reading higher compared to the 2nd and 3rd which is theoretically unacceptable, but as MTT assay is highly sensitive and when the plate is read in microplate reader sometimes it

takes the color due particles as the formazan product and gives the high readings. So this was one of the errors encountered in the colorimetric test.

4.2 Cytotoxicity based on nanoparticle composition

In this experiment 6 samples were fabricated by varying the proportion of cobalt ferrite and nickel ferrite while synthesizing nanocomposite spheres. The nanocomposite spheres were fabricated with 5%, 10%, and 15% of cobalt ferrite and nickel ferrite encapsulated in PLGA. When the cytotoxicity test was conducted it proved that the sample with high percentage of nanoparticles effected the cell growth and resulted in increased cell death. Shown in Figure 26 and 27, samples with 5% magnetic nanoparticles are less toxic compared to a 10% magnetic nanoparticles sample which in turn is less toxic compared to sample with 15% magnetic particles, i.e. samples with 5%> 10%>15% magnetic nanoparticles.

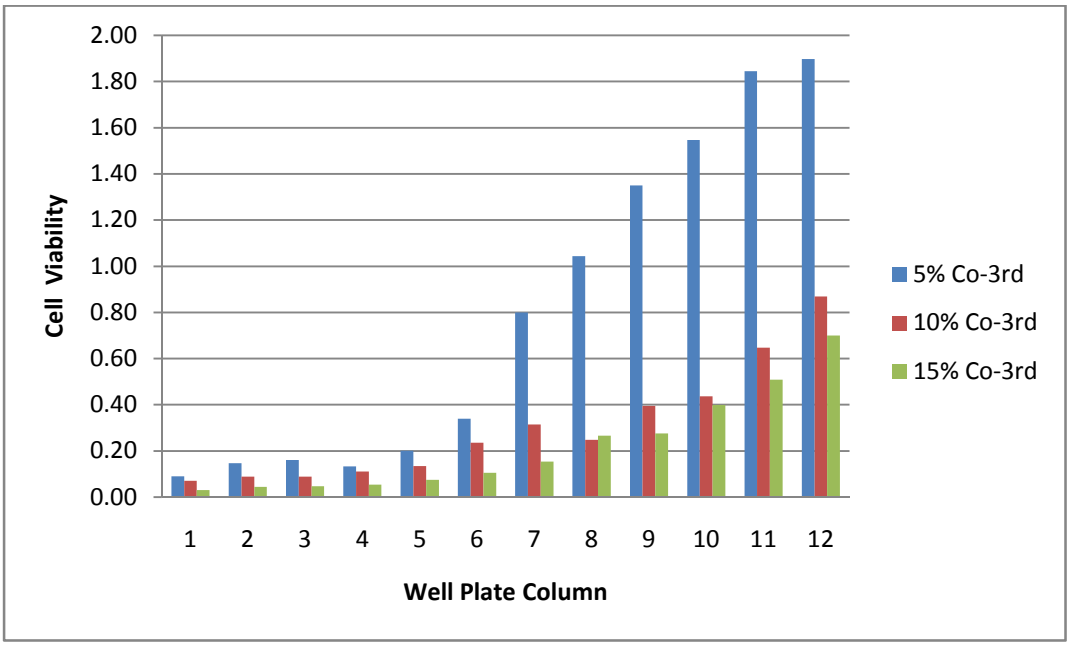


Figure 26: Comparison between cobalt ferrite samples from 3rd day readings.

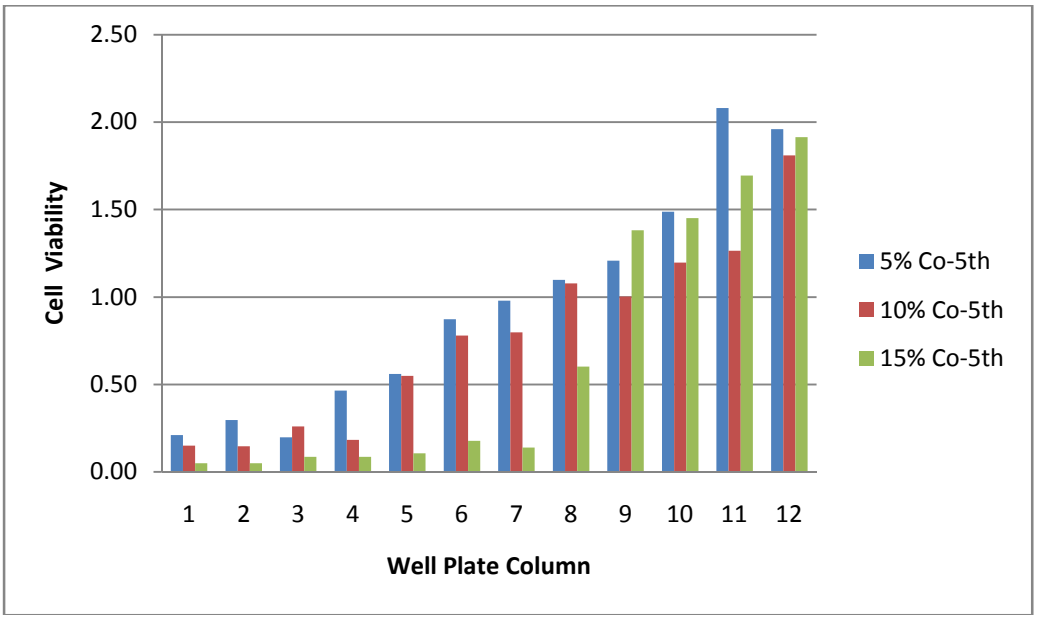


Figure 27: Comparison between cobalt ferrite samples from 5th day readings.

From the graphs plotted from the 3rd day and 5th day readings from the microplate reader, its known that the cell proliferation rate was increased from 3rd to 5th day and also the samples with low magnetic nanoparticles favored cell growth, compared to higher nanoparticles composition.

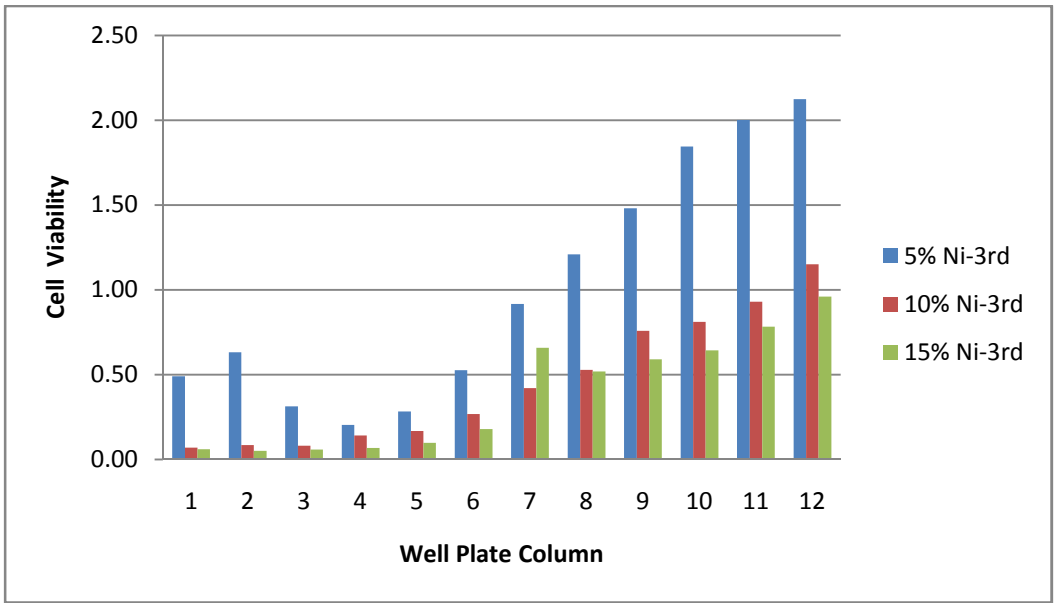


Figure 28: Comparison between Nickel Ferrite samples from 3rd day readings.

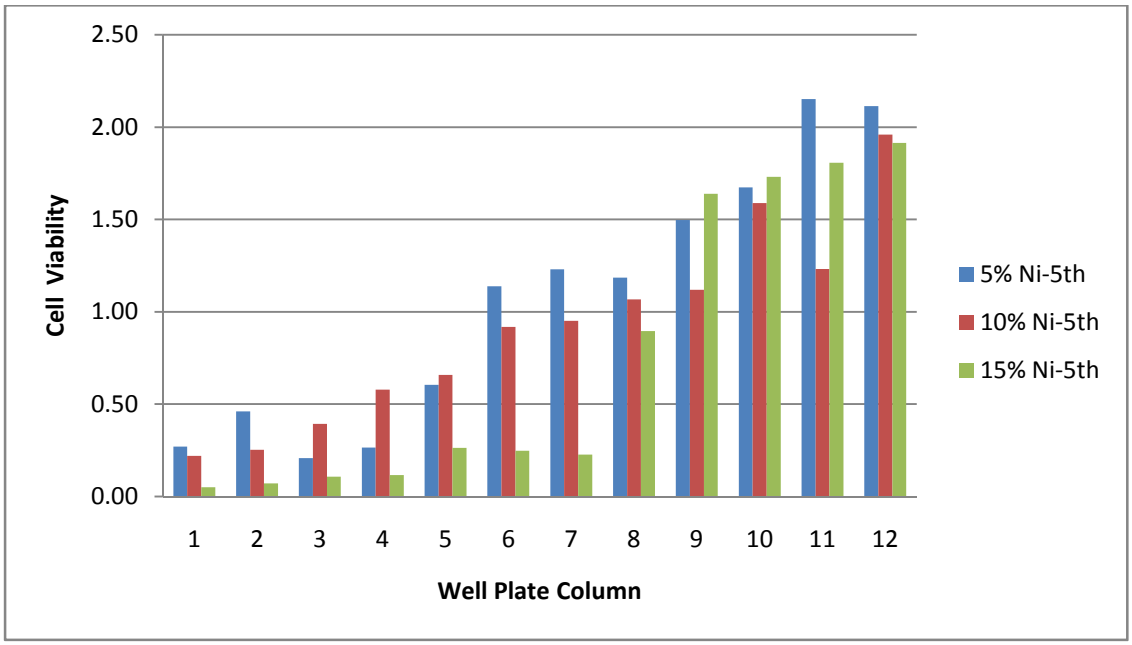


Figure 29: Comparison between Nickel Ferrite samples from 5th day readings.

Plots were drawn for comparison between nanocomposite spheres with 5%, 10%, 15% nickel ferrite composition. As shown in Figures 28 and 29, the same result was observed showing the increase in cell growth from 3rd day to 5th day and more cell viability with the samples having low nanoparticles composition. Some errors in readings have been encountered due to the high sensitivity of the MTT assay, showing some fluctuations at one or two readings showing that the higher composition sample has highest reading compared to lower composition and, air bubbles in the well can also result in higher readings causing an error in the output.

The aspect of cytotoxicity due to nanocomposite spheres was tested to find:

- The toxicity of the sample based on varying the cobalt ferrite and nickel ferrite composition within the nanocomposite spheres.
- The toxicity of same sample by changing the concentrations among the columns in the microplate

- The comparison between the different nanoparticles at same compositions, for example to find the cell viability for sample with 5% cobalt ferrite and sample with 5% nickel ferrite.

4.3 Cytotoxicity based on varying concentration of same sample

The effect of changing the nanoparticle composition along with their concentration on the cells was studied .cobalt ferrite and nickel ferrite were exposed to mouse microphage at different concentrations each sample with the highest concentration being 500 µg/ml and their by reducing it to half i.e.250 µg/ml and so on with the lowest concentration being 0.24 µg/ml and then they were incubated for 3rd day and 5th day readings. After incubating the cells for 3 days and 5 days with these sample concentrations, the cells which are exposed to higher concentration of sample died at a high rate, with the highest cell growth observed at the cells exposed to lower concentration of nanoparticles. This proves that cell viability increased with decrease in concentration.

4.4 Cytotoxicity varies based on nanoparticle

Graphs in Figure 30 and 31 are plotted for the comparison between the samples with same composition but different nanoparticles, namely cobalt ferrite and nickel ferrite. Culture medium was assumed to have maximum cell viability under same conditions at which cells exposed to nickel ferrite and cobalt ferrite are cultured.

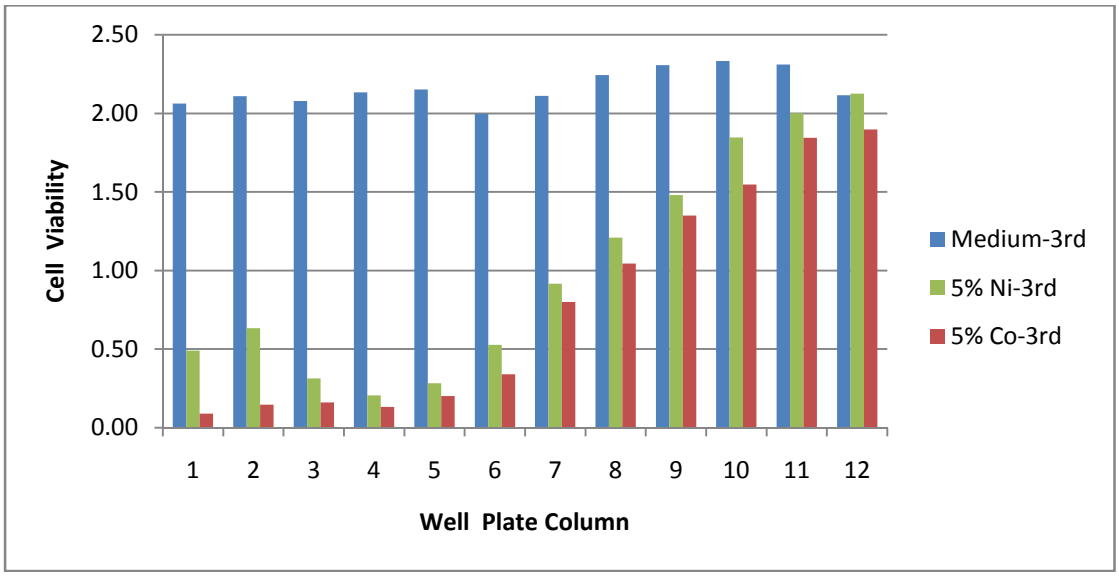


Figure 30: Comparison between 5% nickel ferrite and 5% cobalt ferrite with reference to culture medium from 3rd day readings.

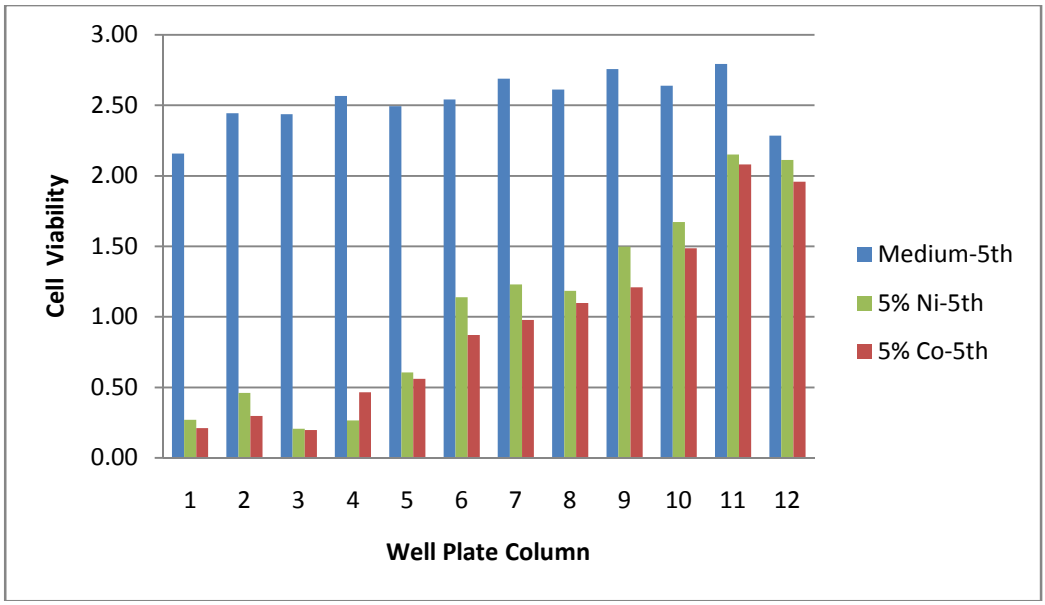


Figure 31: Comparison between 5% nickel ferrite and 5% cobalt ferrite with reference to culture medium from 5th day readings.

From the experiment we believed that, even at lower composition nickel ferrite was less toxic compared to cobalt ferrite. Similar comparison was made between samples made of 10%

cobalt ferrite and 10% nickel ferrite along culture medium as a reference and the graphs plotted are shown in figure 32 and 33.

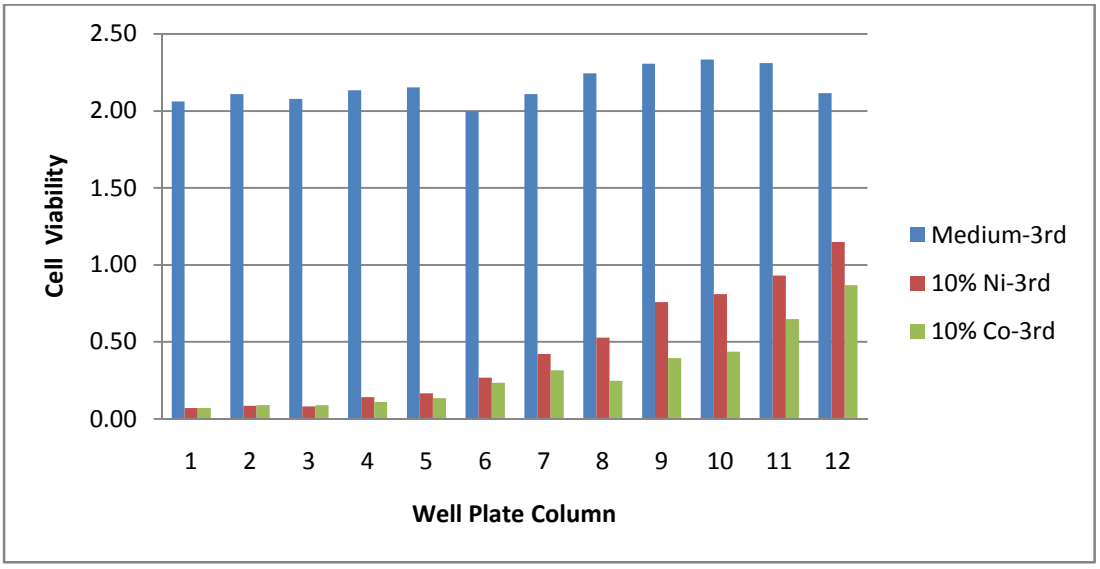


Figure 32: Comparison between 10% nickel ferrite and 10% cobalt ferrite with reference to culture medium from 3rd day readings.

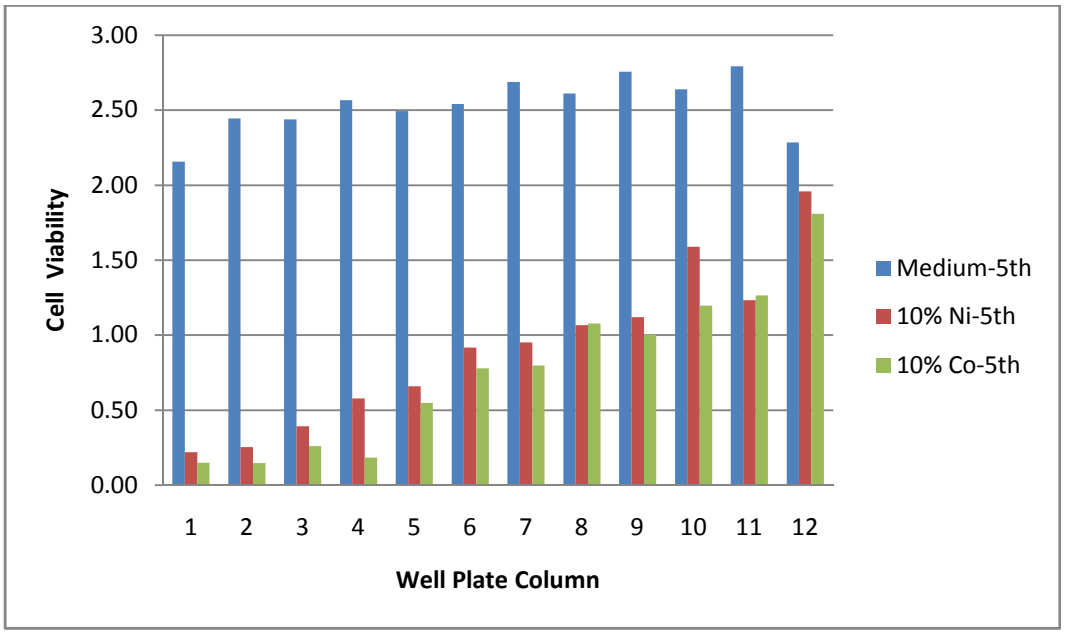


Figure 33: Comparison between 10% nickel ferrite and 10% cobalt ferrite with reference to culture medium from 5th day readings.

Graphs in figure 34 and 35 are plotted in reference to the results from the samples made of 15% cobalt ferrite and 15% nickel ferrite. At all three tested compositions of cobalt ferrite and nickel ferrite, nickel ferrite was observed to be less toxic than cobalt ferrite. But nickel ferrite also effects the cell viability; almost 80-85% of the cells were killed at higher at concentration 500 µg/ml in all three samples.

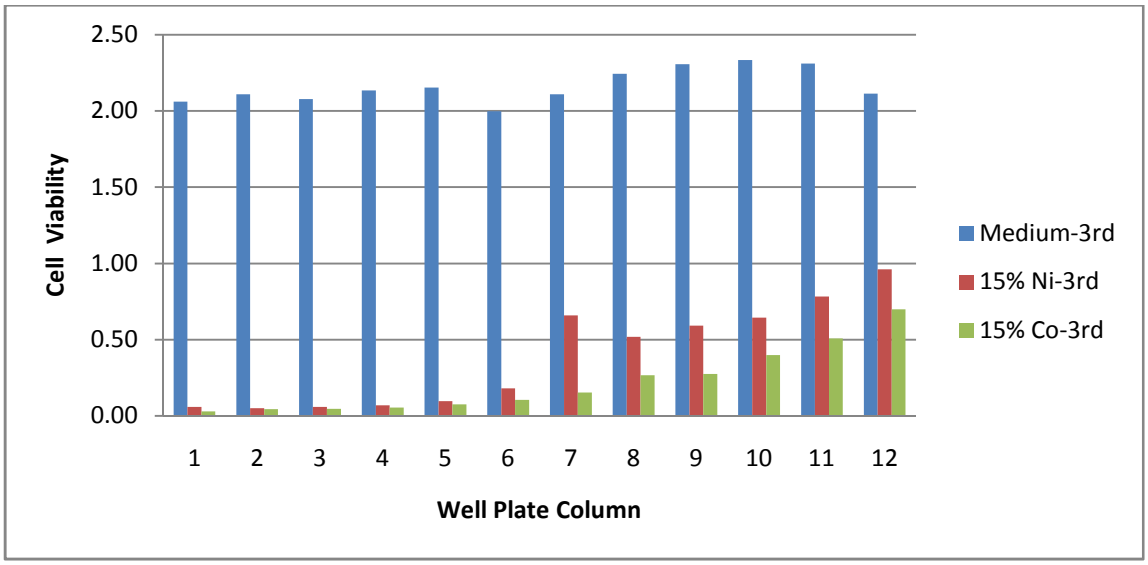


Figure 34: Comparison between 15% nickel ferrite and 15% cobalt ferrite with reference to culture medium from 3rd day readings.

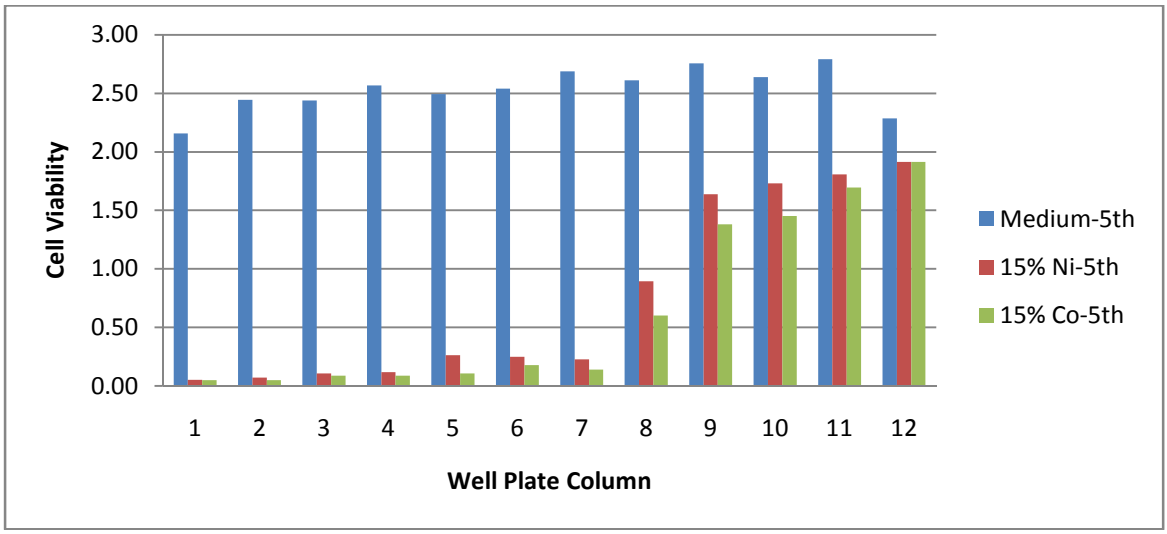
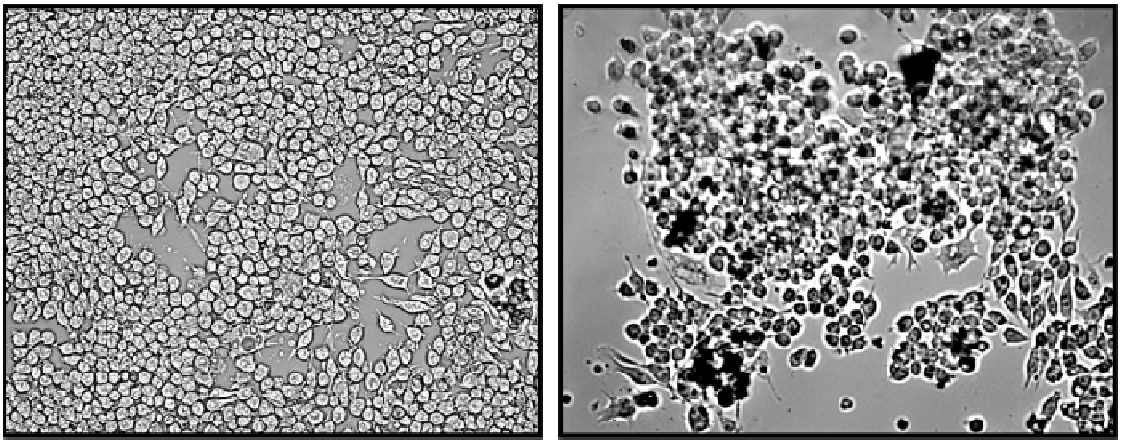


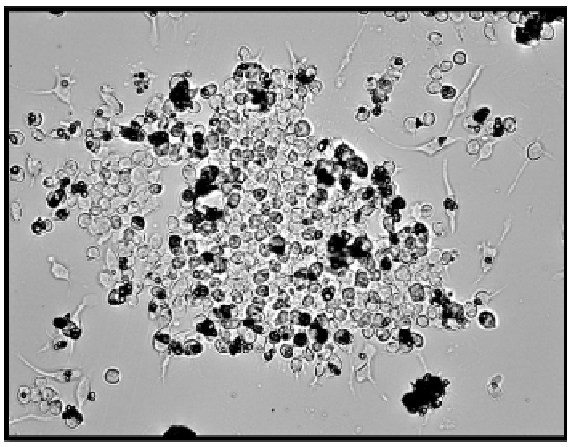
Figure 35: Comparison between 15% nickel ferrite and 15% cobalt ferrite with reference to culture medium from 5th day readings.

In all the above plots derived from microplate readings, cell death or the cytotoxicity was increasing with reducing concentration of the sample and with reduced composition of nanoparticles. Nanocomposite spheres fabricated from nickel ferrite are less toxic compared to spheres from cobalt ferrite. Fluctuations in the readings from the Elisa reader can be due to some errors including air bubbles; color from sample which can be read as formazan by the reader and the process is semi-automatic so, manual interruption in the process can affect the results. In any comparison among the three aspects, it was observed that the cell viability has increased from the 3^{rd} day to the 5^{th} day.

Figures 36 are the microscopic images of the mouse microphage; these images were taken to find the interaction of nanocomposite spheres with the raw cells. While conducting the cytotoxicity test, same number of cells were grown on 8 well chamber slide and incubated along with the microplate, the following day magnetic nanocomposite spheres were added to the slide at concentration 500 µg/ml and incubated for 3days for the microscopic images, it can be noticed from the images that compared to image (a) in figure 36 most of the cells died by 3^{rd} day and very few were observed by 5^{th} day image with the dark color clusters in image (b) & (c) being the nanocomposite spheres.



(a) (b)



(c)

Figure 36: Microscopic images of mouse raw cell (a) before, (b) after exposing for 3 days and (c) for 5 days to magnetic nanocomposite spheres (500 µg/ml).

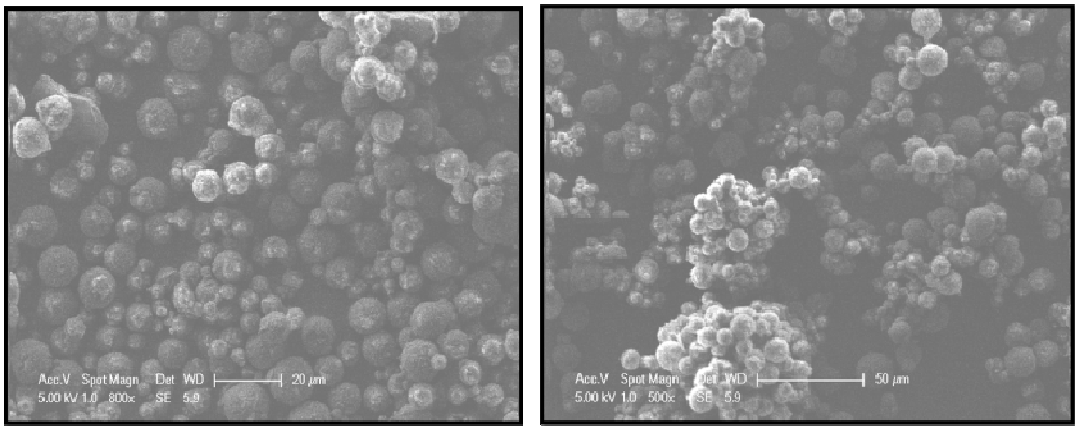


Figure 37: SEM images of Nanocomposite spheres

Size and morphology of the magnetic nanocomposite spheres was important as it determines the release, degradation behavior, and efficacy of the therapeutic agent effecting the cellular uptake and tissue penetration. So, for finding the morphology of the particles SEM was used (shown in figure 37). Agglomeration was observed in the SEM images which might be due magnetic attractions which can effect in two different ways firstly if agglomerated nanoparticles are encapsulated in PLGA, secondly if the nanocomposite gets agglomerated during the fabrication process. Unfortunately due to the agglomeration issues the size of the particles fabricated in the lab were beyond the acceptable limit at size around 1- 2 µm.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

In this report fundamentals of the nanotechnology along with its fabrication methods and types of nanomaterials were discussed. Nanotechnology being widely researched these days, pharmacists and biomedical industry are conducting interesting studies on using the nanotechnology for medical purposes where the ordinary drug delivery cannot be efficient and for applications that need constant administration. Some studies and efforts have succeeded in utilizing the nanomaterials by taking advantage of their small size and surface area. So, among the nanomaterials there are many forms that can be helpful in particular application. Among them dendrimers and polymer micelles are being used with lot of research being done to modify their properties to be more perfect for the application. As the human body has a very good immune system, that constantly protect body from any unknown foreign particles, it's been difficult to fabricate a structure that is compatible chemically as well as physically to the body. Study was made on these different nanomaterials being used with more emphasis on magnetic nanocomposite spheres.

Among many magnetic materials available, cobalt ferrite and nickel ferrite were of great interest because of their excellent magnetic properties such as good saturation magnetization and coercivity; Ferrites on the other hand are more compatible to the body among the metal oxides available. Cobalt ferrite and nickel ferrite were synthesized using sol-gel process and these metal oxides were encapsulated in PLGA to be less toxic and harmless to the normal cells in the body. As metal oxides are being tested for cytotoxicity, the composition of metal oxide and polymer were varied to find the level of toxicity. Six samples were fabricated from cobalt ferrite and

nickel ferrite for comparison. Cytotoxicity test was conducted using standard colorimetric test with MTT assay for 3rd day and 5th day observation. Raw cells which are mouse microphage are tested for toxicity. Readings from Elisa reader was clear that nickel ferrite was less toxic than cobalt ferrite even at low composition; Nanocomposite spheres affected the cell viability. Cell death due to nanocomposite spheres reduced with the decrease in sample concentration and the results were plotted.

5.2 Future Work

- Surface modifications need to be made to the nickel ferrite and cobalt ferrite nanoparticles to prevent agglomeration and reduce toxicity.
- Changes need to be made in fabrication of nanocomposite spheres to prevent residues and loss of particles by the end of process.
- New magnetic materials need to be investigated for drug delivery as cobalt ferrite and nickel ferrite are found to be toxic to raw cells.
- Cytotoxicity test needs to be conducted for different cell lines because different cell lines respond differently to nanoparticle exposure.

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