Bioprinted Superparamagnetic Nanoparticles for Tissue Engineering Applications:
Synthesis, Cytotoxicity Assessment, Novel Hybrid Printing System

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ABSTRACT

Bioprinted Superparamagnetic Nanoparticles for Tissue Engineering Applications: Synthesis, Cytotoxicity Assessment, Novel Hybrid Printing System
Kivilcim Buyukhatipoglu

Novel technologies are required in tissue engineering to manufacture three-dimensional organs with complex architecture. While superparamagnetic nanoparticles have been widely used in medicine for magnetic resonance imaging and targeted drug delivery, they have not been extensively applied in tissue engineering. These nanoparticles would allow active patterning and non-destructive imaging during tissue growth and development. However, no inexpensive method exists for synthesis of commercial amounts of these nanoparticles with controlled morphology, chemistry and size. Furthermore, superparamagnetic nanoparticle cytotoxicity mechanisms are not well understood, which makes it difficult to control or block adverse nanoparticle effects on human health.

In this dissertation, superparamagnetic iron oxide nanoparticles were produced by flame synthesis using a coflow diffusion flame. Nanoparticle flame synthesis has significant advantages, including improved nanoparticle property control and commercial production rate capability with minimal post-processing. Final iron oxide nanoparticle morphology, elemental composition, and particle size was controlled by changing flame configuration, flame temperature, and additive loading, and morphology, elemental composition, and particle size of the synthesized nanoparticles were analyzed by electron microscopy (TEM, ESEM, EDS), and Raman Spectroscopy. Then flame synthesized iron oxide nanoparticle interaction with
endothelial cells was compared to commercially available iron oxide nanoparticles. Flame synthesized particles showed no statistically significant toxicity difference from commercially available nanoparticles, as measured by Live/Dead assay, Alamar blue, and lactase dehydrogenase release. Both synthesized and purchased nanoparticles localized inside the cell cytoplasm as shown by TEM images. Iron oxide nanoparticles resulted in an increase in reactive oxygen species (ROS) formation in cells within the first three hours after nanoparticle uptake, and this ROS formation contributed to actin cytoskeleton disruption. Finally, a new hybrid nano-bioprinting technique that facilitates manipulation and tracking of cells and bioactive factors within a three-dimensional tissue construct was developed. This technique combined the initial patterning capabilities of syringe-based cell deposition with the active patterning capabilities of superparamagnetic nanoparticles. Superparamagnetic iron oxide nanoparticles, either in the alginate biopolymer or loaded inside endothelial cells, were bioprinted using the hybrid solid freeform fabrication direct cell writing system and they were manipulated using an external magnet and imaged by MicroCT.
CHAPTER I: INTRODUCTION

1.1 Thesis overview

Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. The magnetic, chemical, mechanical, optical and electrical properties of materials change as their size approaches the nanoscale and as the percentage of atoms at the material surface becomes significant. Recent developments in nanotechnology allow us to produce, characterize and change functional properties of nanoparticles for use in catalysts [1], gas sensors [2], optical magnetic recording [3] and various biomedical applications including magnetic resonance imaging (MRI), hyperthermic treatment for malignant cells, targeted drug and gene delivery, and magnetic cell separation[4]. With the availability of these new applications, new techniques should be developed to scale the processes to commercially accepted levels at the same time develop lower cost approaches to nanoparticle synthesis.

Many experts worry, however, that the unique properties of these particles could make them toxic, and fear over the potential dangers of nanoparticles has led to increasing calls for tests and regulations. To decrease the potential health risks, nanoparticle toxicity and molecular mechanisms triggered when particles come into contact with living cells should be investigated. By testing engineered particles on cell cultures, researchers could identify those particles that are most likely to be dangerous.

Recent studies have enabled the use of nanoparticles in medicine to unlock new frontiers in diagnosing, treating, and preventing disease; relieving pain; and preserving
and improving human health. In the relatively near term, nanomedicine can address many important medical problems by using nanoscale-structured materials and simple nanodevices that can be manufactured today. Among other bioapplications, nanotechnology has potential to create revolutionary products in regenerative medicine and tissue engineering. There will be a big demand for nanoparticles and nanostructures in tissue engineering, especially in areas such as cell therapy and organ regeneration. Nanoparticles can provide the controlled release of bioregulating signals to tissue scaffold material, can modify material transport properties, or can be used to manipulate cells and bioactive factors in three dimensional tissue scaffolds.

In support of these concepts, this thesis consists of a series of studies to develop a new technique to synthesize superparamagnetic iron oxide nanoparticles in an easy to scale up and affordable method, to investigate the toxic effects and toxicity mechanisms when nanoparticles interact with cells, and finally to explore possible unique applications of the synthesized nanoparticles in tissue engineering. The specific aims of this work are:

- **Chapter 2**: Develop a novel flame synthesis system to synthesize superparamagnetic iron oxide nanoparticles with controlled size and morphology.
- **Chapter 3**: Determine if flame synthesized nanoparticles are equally biocompatible as commercially available nanoparticles, and clarify the underlying nanoparticle toxicity mechanisms in cells.
- **Chapter 4**: Develop a novel hybrid bioprinting system which combines the initial patterning capabilities of syringe-based cell deposition with the
active patterning capabilities of superparamagnetic nanoparticles to facilitate manipulation and tracking of cells and bioactive factors within a three-dimensional tissue constructs.

This thesis advances knowledge in nanoparticle flame system by introducing a unique technique to synthesize bulk iron oxide nanoparticles in a cheap and easy to scale up process. Our research further defines flame synthesis parameters that control the size and chemistry of the synthesized nanoparticles. It also clarified the major toxicity mechanisms that trigger cell death in magnetically labeled nanoparticles, reactive oxygen species formation and actin cytoskeleton disruption. The newly developed hybrid solid freeform fabrication technique could dramatically enhance our understanding of organ development and our ability to fabricate organs in vitro by allowing tracking and manipulation of cells and bioactive factors within the tissue engineering construct.

1.2 Superparamagnetic Nanoparticles

1.2.1 Nanoparticles

A particle having one or more dimensions of the order of 100 nm or less is called a nanoparticle. Extensive nanoparticle libraries, composed of an assortment of different sizes, shapes, and materials, and with various chemical and surface properties, have already been constructed. Carbon nanotubes, liposomes, nanoshells, dendrimers, quantum dots, spherical nanoparticles, and nanorods can be counted as main classes of the existing nanoparticles [5]. Nanoparticles are of great scientific interest since they form a bridge between bulk materials and molecular structures. Material properties change as material
size approaches the nanoscale and the percentage of atoms at the material surface becomes significant. While the percentage of surface atoms in bulk materials larger than one micrometer is microscopic relative to the total atom number, the material surface dominates bulk properties in nanoparticles.

1.2.2 Unique Properties of Nanoparticles

Nanoparticle size confers unique, size-dependent properties such as surface plasmon resonance in some metal particles [6], quantum confinement in semiconductor particles [7] and superparamagnetism in magnetic materials [8]. Copper nanoparticles smaller than 50 nm are super hard compared to bulk copper, and they do not exhibit the same flexibility and ductility [5]. This material property change occurs because bulk copper bending occurs with copper atoms/cluster movement at about the 50 nm scale. When nanoparticles are smaller than 50 nm, there are no clusters to bend. The quantum confinement effect can be observed once the semiconductor nanoparticle diameter is of the magnitude as the wavelength of electron wave function [9]. Quantum confinement describes how the electronic properties, in other words the organization of energy levels into which electrons can climb or fall, and optical properties change when the material sampled is 10 nanometers or less. When nanoparticles are small enough to restrict their electrons and produce quantum effects, they often have unexpected visual properties like gold nanoparticles appearing red in solution. Reactions take place at the surface of a chemical or material; the greater the surface for the same volume, the greater the reactivity. Since nanoparticles have very high surface area to volume ratio, they are
highly reactive. One prime example of surface area to volume ratio at the nanoscale is gold nanoparticles. At the macroscale, gold is an inert element, meaning it does not react with many chemicals. At the nanoscale, gold nanoparticles become extremely reactive and can be used as catalysts to speed up reactions [10].

1.2.3 Superparamagnetism

A unique property of nanoparticles observed only in the nanoscale is superparamagnetism in magnetic materials. Below approximately 15 nm, particles do not show any ferromagnetic behavior, which means particles do not show any permanent magnetization after the external magnetic field is removed. However, they still exhibit strong paramagnetic properties with a very large susceptibility (the degree of magnetization in a substance in response to an applied magnetic field).

Nanoparticles are superparamagnetic rather than ferromagnetic due to the of electron spin alignment under the applied magnetic field. Ferromagnetic particles have unpaired electron spins, which align themselves spontaneously. For this reason, these materials exhibit magnetization without being in a magnetic field. Ferromagnetic particles also exhibit permanent magnetization when they are removed from the magnetic field. When the field direction is swapped, ferromagnetic materials initially oppose the field change but eventually most domains switches their magnetization vectors and the same inverse magnetization is accomplished. When ferromagnetic materials reach particle dimensions smaller than a particular domain, they are no longer ferromagnetic and are called superparamagnetic [11]. In the case of paramagnetic particles, a magnetic
field is altered by the magnetic materials present in it. If a particle contains magnetic moments that can be aligned in an external magnetic field, this will amplify the field. Such substances exhibit the property of paramagnetism. In contrast to ferromagnetic materials, paramagnetic materials do not exhibit any permanent magnetization when they are removed from the magnetic field. In paramagnetic materials, permanent atomic magnetic moments can be reoriented in an external field. Orbiting electrons or atomic nuclei are the source of these magnetic moments. When an external magnetic field applies torque to these moments they orient parallel to the field [12].

Superparamagnetism enables nanoparticle stability and dispersion upon magnetic field removal, as no residual magnetic force exists between the particles. Apart from the application areas of superparamagnetic nanoparticles in medicine, which will be described later in detail, they are used as ferrofluids in industry to form liquid seals around the spinning drive shafts in hard disks, to reduce friction, and to remove heat from the voice coil in loudspeakers [13].

1.3 Superparamagnetic Nanoparticle Synthesis

Nanoparticle synthesis methods can be varied to improve control over particle size, distribution and morphology to synthesize cheaper nanoparticles with enhanced functionality. The superparamagnetic nanoparticles widely in medicine are commonly synthesized using two methods: liquid phase synthesis and gas phase synthesis. Liquid phase synthesis methods include sol-gel processing, coprecipitation, inverse microemulsions and polymer matrix-mediated synthesis. Gas phase synthesis methods
include laser ablation, plasma synthesis, chemical vapor decomposition and combustion synthesis.

1.3.1 Liquid Phase Synthesis Methods

Sol-gel processing, a wet chemical synthesis approach, generates magnetic nanoparticles in a multistep process by gelation, precipitation, and hydrothermal treatment [14]. The process starts with a chemical solution which acts as the precursor for discrete particles. Metal alkoxides and metal chlorides are the common precursors, and they undergo various forms of hydrolysis and polycondensation reactions. Metal oxides form by connecting the metal centers with oxo (M-O-M) or hydroxo (M-OH-M) bridges. This solution forms a gel-like system containing both liquid phase and solid phase morphologies including discrete particles. The remaining solvent is removed by a drying process, and this result in significant gel shrinkage and densification. During the solvent removal process, imposed structural template changes influence the ultimate nanoparticle microstructure. A thermal treatment process is often necessary to obtain further polycondensation and enhance mechanical properties and structural stability. Size distribution of semiconductor, metal, and metal oxide nanoparticles can be manipulated by either dopant introduction [15] or heat treatment [16]. Dong and Zu [17] synthesized α-Fe₂O₃ using sol-gel synthesis from Fe(III) salts. First, an aqueous solution of FeCl₃ and a mixture of ethylene oxide (EO) and absolute ethanol (EtOH) were cooled in an ice bath. Then a mixture of EO and EtOH was slowly dropped into an aqueous solution of FeCl₃. A red–brown sol formed first for all the samples, and some of the sol became a gel. The
sols and gels were heated at 200°C to drive out by-products. After further heating at 300°C, Fe₂O₃ nanoparticles were obtained.

Another wet chemical iron oxide nanoparticle synthesis method is coprecipitation of Fe²⁺, Fe³⁺ aqueous salt solutions by addition of a base [18-21]. Fe²⁺ and Fe³⁺ ratio, media pH and ionic strength, and salt type such as chlorides, sulphates, nitrates, perchlorates control final nanoparticle size, shape and composition. When a base is added to an aqueous mixture of Fe²⁺ and Fe³⁺ chloride at 1:2 molar ratio, magnetite is obtained. The color of the precipitated magnetite is black. The overall chemical reaction can be written as follows:

\[
\text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^- \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O} \tag{1}
\]

This method is also widely used to synthesize other nanoparticles like CdSnO₃ semiconducting oxide [22], ZnS and CdS particles [23] and Mn-doped ZnO nanocrystalline particles [24].

In the inverse microemulsion method, two inmiscible liquids (usually, water and oil) and a surfactant form the microemulsion system. Droplets of water-in-oil or oil-in-water are stabilized by surfactants when small amounts of water or oil are used, respectively. The droplet size can be precisely controlled in the nanometer range by changing the water or oil to surfactant ratio. These nanodroplets, containing precursor magnetic nanoparticle ions, can be used as nanoreactors to carry out the chemical reactions required for particle nucleation. Particle growth occurs by recruiting precursor ions and precipitate molecules from droplets void of particles [25, 26]. Chin and Yaacop [27] used a water in oil microemulsion system to prepare magnetic iron oxide
nanoparticles. They were able to synthesize 10 nm diameter spherical superparamagnetic iron oxide nanoparticles.

Another wet chemical synthesis method to synthesize magnetic nanoparticles is polymer matrix-mediated synthesis [28]. In this method nanoscale magnetic particles use a rigid structure as a host or matrix for growth. Organic resins, polymers, zeolite and mesoporous solids have been used as host materials. The host provides sites for nanoparticle nucleation and controls the final synthesized nanoparticle size [29]. Radhakrishnan et al. [30] synthesized the ferric oxide nanoparticle powder by first forming a complex of ferric chloride (FeCl₃) with polymers of polyethylene oxide, polyacrylamide and polyvinyl pyrrolidone then reacting the same with stoichiometric proportions of ammonium hydroxide or sodium hydroxide.

1.3.2 Gas Phase Synthesis Methods

In vapor-phase nanoparticle synthesis, conditions are created where the vapor phase mixture is thermodynamically unstable relative to solid nanoparticle formation. Laser ablation is a common nanoparticle gas synthesis method. A laser beam is used to evaporate material from a target material into a surrounding gas, where it condenses to form particles [31, 32]. Ablation experimental conditions, including laser wavelength, power, gas type and gas pressure, can be changed to control the final nanoparticle size and morphology. In plasma nanoparticle synthesis, microwaves generate plasma by gas ionization, which transfers the heat necessary for vaporization and chemical reactions to occur [33-35]. Plasma temperatures as high as 1000°C can be achieved using this
technique, leading to fast chemical reactions forming nanoparticles. In this method base pressure, gas pressure and RF power can be modified to change the final nanoparticle properties. In chemical vapor condensation, vapor phase precursors are brought into a hot-wall reactor under conditions that favor particle nucleation in the vapor phase rather than film deposition on the wall [36, 37]. In this process, the most important process parameters determining the quality and usability of the nanopowders are the total pressure (typical range from 100 to 100,000 Pa), the precursor material (decomposition kinetics and ligands determining the impurity level), the partial pressure of the precursor (determining the production rate and particle size), the temperature or power of the energy source, the carrier gas (mass flow determining the residence time) and the reactor geometry.

Another vapor phase nanoparticle synthesis method, which is used in this dissertation, is combustion (or flame) synthesis. Flame synthesis is widely used for the manufacture of bulk nanoparticle quantities. Silica, titania and carbon black nanoparticles are predominately manufactured using flame synthesis with typical production rates of 100 metric tons per day and annual production rates of several million metric tons. These powders are used as reinforcing agents, opacifiers, pigments, and in optical fiber fabrication [38]. In recent years investigators have re-examined combustion synthesis techniques as a means to engineer advanced materials designed at the microscopic level. In particular, gas-phase combustion synthesis has been extended to production of particles for use in high-temperature superconductors [39], electronic substrates [40], and catalytic applications [41, 42].
To understand how combustion synthesis works, it is necessary to understand particle formation and growth in the flame. Precursor material is injected in the burner as a gas, droplets or solid particles. When liquid or solid precursors are exposed to high flame temperatures, they evaporate into vapors that in turn react to form monomers. Monomers then cluster, and primary particle homogeneous nucleation occurs. The primary particles collide, coalesce, and provide surface growth sites. When the characteristic coalescence time is of the same order as the characteristic collision time, branchy agglomerates of primary particles are formed. These agglomerates are physically held together by weak bonds which can be readily broken. Nucleation, deposition, coalescence and agglomeration processes are strong functions of system conditions such as temperature, pressure, burner configuration, and reactant concentrations. When branchy agglomerates are exposed to high temperatures, or long residence times, they completely collapse or coalesce to form uniform individual particles with larger characteristic dimensions [43].

Gas phase combustion synthesis, or flame synthesis, has significant advantages over liquid phase synthesis processes. Gas grown materials generally possess higher purity levels compared to liquid or solid state processes. In addition, flames produce self-purifying high temperatures and the heat of combustion activates precursor pyrolysis, hydrolysis, precursor droplet vaporization, and oxidation. Combustion synthesis allows control of particle size, size distribution, phase and composition by altering flame operating conditions such as temperature, reactant concentration, stoichiometry, pressure, turbulence, burner configuration, precursor injection location, particle collection location,
supplementary laser irradiation, and external electric fields among others. Flame synthesis usually occurs as a single step process, whereas wet chemical methods take multiple steps. The flame synthesized final product requires no subsequent post-processing such as washing, and this solvent free processing leads to less process waste. Most importantly, combustion synthesis is an easily scalable process that can achieve high product yields and large, continuous production rates [44]. Flame technology has high potential for inexpensive nanoparticle manufacturing. For instance flame synthesis produces the cheapest submicron powder, titania, on the market today ($1/lb, including finishing cost) [43].

Combustion synthesis has previously been used to produce iron oxide nanoparticles. Janzen and Roth [45] synthesized 4-12 nm iron oxide nanoparticles via a gas phase route using a low pressure H₂/O₂/Ar flame. Iron pentacarbonyl (Fe(CO)₅) at concentrations from 262 to 2096 ppm was the precursor material. Particles were analyzed in situ according to their mass and charge by means of a particle mass spectrometer (PMS). BET-absorption and transmission electron microscopy (TEM) was used to determine particle surface area, size, and structure ex situ. Experimental results were verified by a theoretical model including the calculations of H₂/O₂/Ar gas flows as well as transport properties of the burner stabilized flame and particle dynamics. The results indicate compact, spherical particles in the size range of 3 to 6 nm, depending on the flow coordinate and the dopant concentration.

Zachariah [46] used a premixed methane/oxygen flame to produce silicon coated iron oxide nanoparticles. Iron pentacarbonyl and hexamethyldisiloxane were used as
magnetic and non-magnetic precursor materials. X-ray diffraction, electron microscopy, Mossbauer spectroscopy and magnetization data measured by SQUID magnetometer showed that 30-100 nm diameter composite particles containing 5-10 nm Fe₂O₃ encased in silica were formed. By controlling precursor loading and flame temperature, the silica coating of individual iron oxide nanoparticles and cluster aggregation rate were controlled. The synthesized composite nanoparticles showed superparamagnetic behavior. However a premixed flame represents a limiting case of co-flow diffusion flames, resulting in fast precursor conversion at high temperatures followed by high collision rates and immediate particle coalescence, which produces larger particles compared to coflow diffusion flames [47].

Xing et al. [48] used a counterflow diffusion flame reactor in which two rectangular channels carrying combustion gases were positioned opposite each other. They used hydrogen as the fuel and oxygen as the oxidizer. Gases exiting each channel imposed upon each other at a gas stagnation plane to synthesize hexagonal and cubic iron oxide nanoparticles with primary size of 40 nm.

Despite these studies, there is a lack of data in the literature showing size and chemistry control of synthesized iron oxide nanoparticles by varying the experimental flame conditions. Synthesizing bulk amounts of iron oxide nanoparticles with an easy to scale up and cheap method is very important if nanoparticles are to be used in various industrial applications or for treating human disease. Combustion synthesis offers unique advantages, however a detailed investigation should be performed to determine the flame
configuration, precursor properties, and gas flow rates which yield optimal iron oxide size, chemistry and morphology.

1.4 Applications of Superparamagnetic Nanoparticles in Biomedical Sciences and Medicine

Superparamagnetic iron oxide nanoparticles, such as $\gamma$Fe$_2$O$_3$ and Fe$_3$O$_4$, have been widely used in biomedical applications and medicine for more than 30 years due to their unique property of exhibiting magnetic behavior only in the presence of a magnetic field [4]. Nanoparticles are used to diagnose and treat diseases ranging from cancer to atherosclerosis to glaucoma. They are used in magnetic resonance imaging, hyperthermia, drug and gene delivery, cell separation and purification and tissue engineering [4].

1.4.1 Coating and Functionalization of Superparamagnetic Nanoparticles

Nanoparticles are often specifically targeted to a biological tissue or cell type. A ligand for a target cell receptor is attached to a coated nanoparticle or carrier-drug conjugate. However, the nanoparticle coating material should be chosen carefully. Nanoparticle coatings composed of both inorganic and polymeric materials have been studied [4]. Polymeric coating materials can be classified as synthetic or natural. Polymers based on polyethylene-co-vinyl acetate, polyvinylpyrrolidone (PVP), polylactic-co-glycolic acid (PLGA), polyethyleneglycol (PEG), and polyvinyl alcohol (PVA), are typical synthetic polymer coatings [49, 50]. Gelatin, dextran, chitosan, and pullulan can be used as natural polymer coatings [51-53]. Various surfactants, such as
sodium oleate, dodecylamine, and sodium carboxymethylcellulose are also usually used to enhance nanoparticle dispersion in an aqueous medium [54, 55].

Gupta and Curtis [56] coated superparamagnetic nanoparticles with PEG through chemical precipitation of ferric and ferrous salts and studied the influence of PEG coated superparamagnetic nanoparticles on human fibroblasts. They found that PEG coated nanoparticles did not affect cell adhesion and cell morphology. They used TEM, SEM and light and fluorescence microscopy techniques to show that uncoated magnetic nanoparticles which were internalized within the fibroblast cytoplasm formed vacuoles while surface modified PEG-coated nanoparticles did not change cell behavior.

Pradhan et al. [57] investigated in vitro toxicity and cellular interactions of lauric acid and dextran-coated magnetite nanoparticles with mouse fibroblast and human cervical carcinoma cells. Lauric acid-coated magnetite nanoparticles were more toxic than dextran-coated magnetite nanoparticles. Cellular uptake of lauric acid-coated magnetic nanoparticles was more than that of dextran-coated magnetite nanoparticles.

Wilhelm et al. [58] synthesized anionic nanoparticles by alkalizing Fe$^{2+}$ and Fe$^{3+}$ salts. These anionic nanoparticles showed a high affinity for the cell membrane and thus were captured by cells with an efficiency three orders of magnitude higher than the widely used dextran-coated iron oxide nanoparticles. The surface coating of anionic particles with albumin strongly reduced non specific interactions with the plasma membrane as well as overall cell uptake. It also restored the ability to induce specific interactions with targeted cells by coadsorption of a specific ligand on the particle surface.
Targeted nanoparticles can be synthesized by binding biological molecules such as antibodies, proteins, and ligands to the polymer surfaces using amide or ester chemical coupling methods [4]. There could be numerous ligands that could be used target cell surface receptors. Some possible candidates for conjugating to nanoparticles are as follows. Transforming growth factor-α (TGF-α) can be used to promote cell proliferation and differentiation and may be important for normal wound healing [59]. Nerve growth factor can be used to promote neurite outgrowth and neural cell survival [60]. Pullulan, a non toxic, non-immunogenic, non-antigenic material, can be used to increase water solubility or to expand the plasma [61]. Elastin can be used as a cross-linking protein in the extracellular matrix that provides elasticity for tissues [62]. Albumin as the major serum protein can be used for binding a wide variety of lipophilic compounds including steroids [63]. Tat peptide can be used for enhancing intracellular delivery and RGD peptide can be used for increasing cell spreading and differentiation [64]. Folic acid as a poorly immunogenic material can be used to target cancer cells [65]. Hydrophilic coating molecules should be attached to nanoparticles using linker molecules such as 1-ethyl-3-dimethylaminopropyl carbodi-imide hydrochloride (EDCI), N-succinimidyl 3-2-pyridyldithio propionate (SPDP), N-hydroxysuccinimide or N, N’ methylene bis acrylamide (MBA) [66]. Gupta [67, 68] synthesized superparamagnetic iron oxide nanoparticles with specific shape and size and coupled them to insulin, lactoferrin and ceruloplasmin for targeting to cell surface receptors. This prevented nanoparticle endocytosis. This suggests that cell response can be directed via specifically engineered particle surfaces. Surface functionalized nanoparticles showed high affinity for cell
surface receptor mainly due to ligand–receptor interactions. Their specific attachment to cell surface offers the opportunity to label the cells with magnetic particles while reducing nonspecific phagocytosis.

Nanoparticles have been widely used to target specific cells in the body. Zhou et al. [69] completed a transmission electron microscopy (TEM) study to investigate the ability of magnetic nanoparticles (MNPs) to target breast cancer cells in mice. MNPs were functionalized using luteinizing hormone releasing hormone (LHRH), whose receptors are expressed in most types of breast cancer cells. LHRH conjugated MNPs were injected intravenously into female nude mice bearing tumors for thirty days. 20 hours after MNP injection these mice were sacrificed. Tumors and periphery organs including livers, lungs and kidneys were collected for analysis. Distribution of nanoparticles in cells was investigated using TEM. They found that dispersive LHRH conjugated MNPs were distributed in tumor cells and cells in lungs and livers. They did not observe any LHRH conjugated MNP in kidney cells. Additionally, LHRH conjugated magnetic nanoparticles had a tendency to aggregate and form clusters in tumor cells and cells in lungs where metastases were developed.

Magnetic nanoparticles have also been used to target specific intracellular organelles. Becker et al. [70] investigated cellular uptake of superparamagnetic Fe₃O₄ iron oxide nanoparticles coated with a lipid bilayer and conjugated to streptavidin–fluorescein isothiocyanate (FITC) for targeting subcellular compartments. They synthesized MNPs by coprecipitation of Fe²⁺ and Fe³⁺ salts with cis-9-octadecenoic acid sodium salt (oleate) which yielded paramagnetic iron oxide cores coated with an oleate
lipid bilayer. Biotin-conjugated MNPs were further functionalized by binding the fluorescent tag streptavidin--FITC. MNPs were effectively taken up into cells and even after the uptake into cells, MNPs showed magnetic activity. Such FITC-MNPs were localized in the lysosomal compartment of cells which suggests a receptor-mediated uptake mechanism.

Bertorelle et al. [71] synthesized magnetic fluorescent nanoparticles with magnetite core using chemical oxidation methods to label living cells. The bifunctional nanoparticles possessed a magnetic oxide core composed of a dimercaptosuccinic acid (DMSA) ligand at the surface which was covalently attached to a fluorescent dye. Fluorescence microscopy and magnetophoresis showed that the nanoparticles exhibited a high cell affinity. Fluorescence microscopy was also used to monitor the magnetic nanoparticle localization patterns inside cells. They observed two types of magnetic labeling: nanoparticle adsorption to the cell membrane and nanoparticle internalization inside the cell. After internalization, nanoparticles were restricted inside endosomes, which are endocytotic pathway vesicles inside the cells roughly 300-400nm in diameter. They demonstrated that endosome movement could be directed inside the cell by external magnetic fields, and small fluorescent chains of magnetic endosomes were formed in the cell cytoplasm in the applied magnetic field direction.

1.4.2 Superparamagnetic Nanoparticles in MRI

Modern MRI techniques to differentiate diseased tissue, tumors, inflammation, and microscopic blood vessels use contrast agents to improve signal intensity [72].
Conventional contrast agents used are either paramagnetic metal ions such as Mn$^{2+}$ and Fe$^{3+}$ or rare earth chelates such as Gd$^{3+}$ [72]. However these agents result in some side effects. For instance, free manganese is known to cause fetal toxicity in the cardiovascular system, central nervous system, lung, and liver [73], while gadolinium causes serious kidney damage [74]. Also, those agents have low circulating times throughout the body, which makes complete MRI analysis difficult [75]. Conventional agents are also only capable of enhancing signal intensity during imaging and they cannot carry therapeutic agents [76]. Magnetic nanoparticles have low toxicity levels and longer circulating time in the body. They can increase MR imaging efficiency by increasing tissue contrast while at the same time working as a targeted drug delivery system. Specifically superparamagnetic iron oxide nanoparticles appear dark on MRI images, making them a highly sensitive contrast agent for detection of vascular tissues such as tumors [77, 78].

1.4.3 Superparamagnetic Nanoparticles in Hyperthermia

In addition to current standard cancer therapies such as surgery, chemotherapy, hyperthermia is also being used in clinical practice [79]. In whole body hyperthermia, the body temperature is set at 41.8 °C [80]. Local heat generation can also be achieved by microwave radiation, capacitive or inductive coupling of radiofrequency fields, implanted electrodes, ultrasound, or by lasers. Alternatively, magnetic nanoparticles can be loaded in the tumor, and tumor can be heated using an external alternating magnetic field. Magnetic nanoparticles are currently being investigated for drug targeting to tumors.
through blood circulation and creating local heating. [81]. For magnetic hyperthermia applications, superparamagnetic nanoparticles were injected into a tumor, and when exposed to an oscillating magnetic field and they kill the cancer cells without further damaging surrounding tissue [82-85].

1.4.4 Superparamagnetic Nanoparticles in Drug and Gene Delivery

Superparamagnetic nanoparticles are widely used to target drugs and genes to the location where they are needed to treat diseases like cancer and atherosclerosis, among others. A major disadvantage of most chemotherapeutic approaches to cancer treatment is the fact that they are non-specific. Therapeutic drugs are generally toxic. When these drugs are systemically distributed by intravenous administration, the drugs attack normal healthy cells as well which contributes to therapeutic side effects. Since the 1970’s, magnetic nanoparticles have been investigated as targeted treatment agents to reduce the systematic side effects of chemotherapy drugs [86, 87]. Total drug administered and drug side effects can both be reduced by targeting the drugs to the locations where they are needed. In nanoparticle targeted drug delivery, the drug-nanoparticle complex is injected intravenously. Drugs can then be targeted to the tumor locations using high gradient, external magnetic fields generated by permanent magnets. Once the magnetic carrier reaches the tumor, the therapeutic agent is released from the magnetic carrier, either via enzymatic activity or through changes in physiological conditions such as pH, osmolality, or temperature [88]. Similar principles were applied to therapeutic gene delivery to
specific targets in vivo [89]. Specifically, gene vectors associated with superparamagnetic nanoparticles were used to deliver genes delivery to target cells [90, 91].

1.4.5 Superparamagnetic Nanoparticles for Cell Separation and Purification

Isolation and separation of specific cells and molecules are vital to many areas of biosciences and biotechnology, and are the most documented and currently the most useful application of magnetic nanoparticles. Various magnetic particles have been developed as magnetic carriers in separation processes including purification and immunoassays [92-94]. In magnetic cell separation, antibodies attached to the magnetic nanoparticles are used to tag cells of interest. The cell sample is processed through a column that generates a magnetic field when placed within the separator instrument, retaining the labeled cells. In some systems a simple magnet placed next to the cell sample directly retains labeled cells within the tube while supernatant is drawn off [95].

1.4.6 Applications of Magnetic Nanoparticles in Tissue Engineering

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [96]. Tissue engineering scaffolds have been made by traditional processes, including solvent casting, freeze-drying, phase separation and gas foaming all of which can produce interconnected microporous scaffolds with a certain degree of oriented pore structure [97]. However, the connectivity created by these techniques is a result of processing variables such as
solvent evaporation and porogen particle contact, rather than a predetermined engineering design. Precise control over internal architecture is extremely difficult. For this reason a new manufacturing technique called solid freeform fabrication was developed to manufacture scaffolds layer by layer and allow improved architectural control.

A broad array of solid freeform fabrication methods have been developed to manufacture products that incorporate living cells into their systems [98, 99]. Syringe-based cell deposition is a form of solid freeform fabrication that performs extrusion/jetting-based processes to build 3D tissue scaffolds layer by layer [100, 101]. Another method called rapid prototyping combines the strengths of injection molding with those of solid freeform fabrication. Three-dimensional pre-seeded implants can be fabricated without custom-tooling, enabling efficient production of patient-specific implants [102, 103]. Inkjet-based cell printing uses a modified computer printer and an ink cartridge, in which cells are suspended separately. The cells were subsequently printed as a kind of ink onto several biopapers made from biocompatible materials like soy agar and collagen gel [104-106]. Microcontact printing generates mammalian cell patterns on porous scaffolds for tissue engineering using replica printing. Cell patterns were transferred directly from topographically patterned stamps onto porous scaffolds or onto fibronectin-coated glass slides. Stamps inked with cell suspensions allowed repeated substrate patterning. This approach enables control of the spatial scaffold invasion, which promotes hierarchical cell organization and controls cell–cell interactions as a step in preserving cell phenotype [107, 108].
Khalil et al. [109], developed a solid freeform direct cell writing system for the freeform construction of 3D tissue scaffolds. The system was designed to operate at room temperature and low-pressure conditions and to deposit living cells, growth factors, or other bioactive compounds in controlled amounts with precise spatial positioning. The multi-nozzle deposition system applied solid freeform fabrication techniques along with computer aided modeling of heterogeneous structures to build tissue scaffolds. The accuracy of the positioning system was 10 μm, though the deposition method and the distance between the nozzle tip and the substrate affected this value. The designed scaffold model was processed by the data processing system and converted into a layered process tool path. The motion control system was driven by the layered manufacturing technique and the material delivery system consisted of multiple nozzles with different types and sizes. This enabled the deposition of specified biopolymers with different viscosities for constructing 3D tissue scaffolds. Four types of the nozzles were used in the system: solenoid-actuated nozzles, piezoelectric glass capillary nozzles, pneumatic syringe nozzles, and spray nozzles, with size ranges varying from 30 μm to 500 μm. The system could continuously extrude biopolymer gels, or form biopolymers in single droplets with picoliter volumes. The multiple nozzle capability allowed the users to simultaneously deposit cells, growth factors, and scaffold materials, thus enabling the construction of heterogeneous scaffolds with bioactive compounds, or establishing functional gradient scaffolds with different mechanical/structural properties in different scaffold regions.
Chang et al. [110] used the solid freeform fabrication-based direct cell writing system developed by Khalil et al. [109] and examined the effect of solid freeform fabrication–based direct cell writing process, focusing on dispensing pressure and nozzle size, on the viability and functional behavior of HepG₂ cells encapsulated within alginate. Their experimental results revealed a process-induced mechanical damage to cell membrane integrity, which caused a quantifiable loss in cell viability when dispensing pressure and nozzle size were changed. The experimental results also showed that a recovery period may be required following direct cell writing process.

However none of the previous techniques used active patterning capabilities of magnetic forces and magnetic nanoparticles. Ito et al. first used magnetic nanoparticles to establish three-dimensional, in vivo-like tissues consisting of various cell types for tissue engineering [111]. Magnetic force was used to construct a heterotypic, layered co-culture system of rat hepatocytes and human aortic endothelial cells (HAECs). Positively charged magnetite cationic liposomes were used to improve nanoparticle uptake inside the cells. These nanoparticles accumulated in HAECs at a concentration of 38 pg of magnetite per cell. Magnetically labeled HAECs specifically accumulated onto hepatocyte monolayers at sites where a magnet (4000 G) was placed and heterotypic, layered constructs were manufactured. This new co-cultured construct significantly enhanced albumin secretion by hepatocytes, when compared with homotypic cultures of hepatocytes or heterotypic co-cultures of hepatocytes and HAECs without magnets.

Perea et al. [112] presented a novel strategy based on the use of superparamagnetic nanoparticles to obtain an endothelial cell lining on the luminal
surface of vascular conduits. These nanoparticle loaded endothelial cells were also detected non-invasively by MRI. Human umbilical vein endothelial cells (HUVECs) were loaded with clinically approved superparamagnetic nanoparticles, and cell viability and eNOS expression were not affected by nanoparticle uptake. An electromagnet was used to deliver magnetically labeled cells onto a PTFE tubular graft and endothelium was detected with a 1.5T MRI scanner. Tubular scaffolds were seeded using magnetic cell delivery technique which enables the non-invasive imaging of the cells from the substrate. This also enables assessment of the quality of cell delivery procedures.

However no studies have been performed in the literature to create 3D tissue engineered scaffolds containing precisely positioned nanoparticles or cells loaded with magnetic nanoparticles.

1.5 Magnetic Nanoparticle Toxicity Mechanisms

Nanoparticles appear to have some toxic effects that are unusual and not seen with larger particles. Two primary mechanisms for nanoparticle toxicity have been proposed: reactive oxygen species (ROS) formation and actin cytoskeleton disruption.

1.5.1 Nanoparticle Induced Reactive Oxygen Species Formation

Nanoparticles increase reactive oxygen species formation when taken inside cells [113]. Reactive oxygen species (ROS) are ions or very small molecules including oxygen ions, free radicals, and peroxides, both inorganic and organic. They are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct
of normal oxygen metabolism and have important roles in cell signaling. However, during times of environmental stress, ROS levels can increase dramatically, which can result in significant damage to cell structures. High ROS levels are known as oxidative stress.

Nanoparticles might result in ROS formation in several different ways. Reactive oxygen species can be generated directly from free radicals on the nanoparticle surface. Transition metal nanoparticles like iron can generate reactive oxygen species by acting as catalysts in Fenton-type reactions that form hydroxyl radicals [114]. Or nanoparticle uptake might alter the mitochondrial functions or physically damage the mitochondrial membrane to contribute to ROS formation and oxidative stress [115, 116].

Several different nanoparticle types were already investigated for ROS induced toxicity. Combustion derived nanoparticles were found to cause inflammation via oxidative stress and activation of redox-sensitive transcription factors [117]. Combustion is considered a source of toxic chemicals and particles [118]. Combustion derived nanoparticles include diesel soot, welding fume, carbon black and fly-ash particulates. Their combustion origin, small size, universal injury mechanism and common translocation properties united them, and they have the potential to result in a range of adverse effects in the lungs and other organs. These nanoparticles all cause oxidative stress as a combined result of their pathogenic mechanism. This oxidative stress can cause inflammation and oxidative compounds in epithelium which might lead to carcinogenesis. Combustion derived nanoparticles are known to be a potential hazard to
the lungs and other systems through the oxidative stress, inflammation and carcinogenesis.

Transition metals have also been shown to induce ROS [119]. They are known to cause inflammation via oxidative stress and activation of redox sensitive transcription factors [120, 121]. They contribute to the observed health effects like fibrosis, chronic inflammatory lung disease, and cancer. When transition metals and polycyclic aromatic hydrocarbons (PAHs) interact with the lining fluids of the lung, they undergo cycling redox reactions that produce ROS [117]. Particles also might result in defects in cell’s genetic material so called genotoxicity. Each particle has its own unique physicochemical characteristic, so mechanisms involved in particle-induced genotoxicity are not clear. Interaction of PAHs with cells may cause genotoxicity and this might cause DNA adducts [122]. Alternatively, ROS induced by the transition metals might also result in DNA strand breakage [123]. Size and iron release from fly ash has been found to lead radical generation and oxidative stress which also result in genotoxicity [124].

1.5.2 Nanoparticle Induced Actin Cytoskeleton Disruption

After MNP endocytosis, the nanoparticles may disrupt the cell cytoskeleton. The cytoskeleton is a dynamic structure that maintains cell shape, protects the cell, enables cellular motion, and plays important roles in both intracellular transport and cellular division. Eukaryotic cells contain three main kinds of cytoskeletal filaments, namely microfilaments (actin filaments), intermediate filaments, and microtubules [125]. Actin filaments are around 5-9 nm in diameter, and they are composed of two tangled actin
chains. Actin filaments are mainly concentrated just beneath the cell membrane, and they are in charge of resisting tension and maintaining cellular shape, forming cytoplasmic protuberances, and participating in cell-to-cell or cell-to-matrix junctions. In relation with these roles, actin filaments are essential to mechanotransduction. They are also important for cytokinesis and, along with myosin, muscular contraction. Actin/myosin interactions also help produce cytoplasmic streaming in most cells [126]. The investigation of actin filament changes is very important in understanding toxicity caused by nanoparticle endocytosis.

There is very limited data representing actin cytoskeletal changes in response to nanoparticle uptake. Gupta and Curtis investigated the effect of PEG coated nanoparticles on the fibroblast cell cytoskeleton compared to the non coated nanoparticles [56]. Their findings showed that in control cells, the microfilaments were well organized in thick bundles forming stress fibers while in cells loaded with non coated nanoparticles the actin fibers were less defined and visibly disorganized in comparison to control cells. They also showed that the microtubules form a dense network equally distributed around the nucleus in the whole cell volume.

1.5.3 Relation between Reactive Oxygen Species Formation and Actin Cytoskeleton Disruption

Although a large amount of evidence indicates potential links between ROS release, actin cytoskeleton disruption, and apoptosis, the actual mechanism which links these processes remains unknown. Current studies propose a number of possible mechanisms which couple changes in actin dynamics to cell death. The regulation of the
voltage dependent anion channel (VDAC) results in the anti-apoptotic activity of gelsolin to occur [127, 128]. The VDAC is a mitochondrial membrane pore which is responsible for sustaining mitochondrial membrane potential and adjusting the release of pro-apoptotic factors [129]. It is known that actin regulates VDAC closure in *Neurospora crassa* [130]. In *Neurospora crassa*, the actin-stabilizing drug phalloidin extended VDAC pore opening and increased ROS levels. Looking at these data F-actin dynamics can be considered to have an important role in cell death by regulating VDAC in eukaryotic cells and by regulating the activity of several ion channels.

Rearranging the cytoskeleton structure in response to stress is a fundamental process in many eukaryotic cells. A less dynamic cytoskeleton cannot respond effectively to such stimuli. If actin disruption plays a role within cell death pathways, a common signaling pathway that contributes to cell viability by remodeling the actin cytoskeleton may be needed. The pathway not only regulates, but is also regulated by, actin dynamics. Constitutive activation of the Ras–cAMP pathway results in mitochondrial dysfunction, which leads to problems in upregulating proteins that protect against oxidative stress, and this decreases cell lifespan. The Ras pathway is also involved in actin regulation [131] and Ras–cAMP pathway and actin may interact to regulate oxidative stress and cell viability [132].

### 1.6 Organization of the dissertation

The central hypothesis of this thesis is that iron oxide nanoparticles can be synthesized with controllable size and morphology by flame synthesis, the synthesized
nanoparticles are equally biocompatible as the commercially available ones, and these iron oxide nanoparticles can be used to manipulate and track cells and bioactive factors within three-dimensional tissue constructs. The thesis is organized as five chapters.

- Chapter I, the introduction chapter, coverings background on superparamagnetic nanoparticles, their applications in medicine and biomedical areas specifically MRI, hyperthermia, drug/gene delivery, cell separation and purification and tissue engineering, and previous nanoparticle toxicity studies and underlying toxicity mechanisms.

- Chapter II describes the combustion synthesis set-up developed for the direct iron oxide nanoparticles synthesis, and control of nanoparticle morphology, composition, and size by varying flame configuration, flame temperature, and additive loading.

- Chapter III presents the comparison of flame synthesized iron oxide nanoparticle interaction with endothelial cells to commercially available iron oxide nanoparticles in terms of toxicity, cellular uptake, cell proliferation. The underlying toxicity mechanisms leading to cell death including reactive oxygen species formation and actin cytoskeleton disruption are explored.

- Chapter IV describes a new hybrid nano-bioprinting technique designed which facilitates manipulation and tracking of cells and bioactive factors within three-dimensional tissue constructs. Cell viability is assessed for various nanoparticle and alginate concentrations, and nanoparticle manipulation inside the tissue scaffolds is demonstrated. The effect of cell dispensing parameters and scaffold
biopolymer characteristics on cell viability, nanoparticle movement, and bioprinting patterning capabilities of the new hybrid system are studied.

- Chapter V concludes the dissertation with the summary of the principle findings and suggested future work.
2.1 Introduction

Superparamagnetic nanoparticle synthesis methods can be grouped into two broad areas: liquid phase synthesis (primarily sol-gel) and gas phase synthesis. Sol-gel processing can be used to generate nanoparticles in a multistep process through gelation, precipitation, and hydrothermal treatment [14]. Iron oxide nanoparticles have been synthesized through wet chemical routes by coprecipitation of Fe$^{2+}$, Fe$^{3+}$ aqueous salt solutions by addition of a base [18-21], polymer matrix-mediated synthesis [28] and inverse microemulsions [25, 26]. Various aerosol processing techniques have been reported to improve the production yield of nanoparticles including plasma synthesis [33-35], laser ablation [31, 32], chemical vapor condensation [36, 37] and spray pyrolysis [133].

Recently, it has been shown that combustion synthesis has significant advantages over liquid phase synthesis processes. Combustion synthesis is the simplest and most economic method for nanoparticle production and is currently used to produce bulk carbon black, titania, and silica powders [134]. The flame synthesized materials generally possess higher purity levels compared to liquid or solid state processes. In addition, flames produce a self-purifying process due to their high temperatures. The heat of combustion activates precursor pyrolysis, hydrolysis, precursor droplet vaporization, and oxidation. Combustion synthesis allows control of particle size, size distribution, phase
and composition by altering flame operating conditions such as temperature, reactant concentration, stoichiometry, pressure, turbulence, burner configuration, precursor injection location, particle collection location, supplementary laser irradiation, and external electric fields among others. Flame synthesis usually occurs as a single step process, whereas wet chemical methods take multiple steps. The flame synthesized final product requires no subsequent post-processing such as washing, and this solvent free processing leads to less process waste. Most importantly combustion synthesis has proven to be an easily scalable process that can achieve high product yields and large, continuous production rates [43, 44, 135].

Iron oxide nanoparticles have previously been synthesized using combustion synthesis. Janzen and Roth [45] synthesized 4-12 nm iron oxide nanoparticles via a gas phase route using a low pressure H₂/O₂/Ar flame. Zachariah et al. [46] used a premixed methane/oxygen flame to produce silicon coated iron oxide nanoparticles. However a premixed flame represents a limiting case of co-flow diffusion flames, resulting in fast precursor conversion at high temperatures followed by high collision rates, immediate particle coalescence, and large particles [47]. Xing et al. [48] used a counterflow diffusion flame reactor in which two rectangular channels carrying combustion gases were positioned opposite each other. Gases exiting each channel imposed upon each other at a gas stagnation plane to synthesize hexagonal and cubic iron oxide nanoparticles with primary size of 40 nm.

Recently, combustion synthesis has been used to produce nano-sized metal oxides of different morphologies by inserting solid substrates into the flame. Lee et al. [136]
used an inverse diffusion flame to synthesize Ni-catalyzed multiwalled carbon nanotubes and nanofibers on a catalytic substrate. The nanomaterial and shape formed were highly dependent on flame temperature. Carbon nanofibers were synthesized at temperatures lower than 900 K, carbon nanotubes were synthesized between 900 and 1400 K, and iron nanorods were synthesized above 1400 K. Flame temperature also affected size for a given nanomaterial and shape, since larger diameter carbon nanotubes were observed closer to the flame. Later studies showed similar elongated nanomaterial synthesis of varied composition using solid substrates. Single crystalline zinc oxide nanowires were produced on zinc-plated-steel substrates using an axi-symmetric inverse jet diffusion flame, again with larger diameter nanowires produced at higher temperature [137]. Similarly, 10 to 100 nm diameter iron oxide nanorods a few microns in length were synthesized in a single step by inserting iron probes into an opposed-flow methane oxy-flame [138]. These new developments in combustion synthesis continue to highlight the importance of combustion parameters on nanomaterial properties.

Flame configuration affects nanoparticle size and morphology. Effect of reactant mixing, precursor chemistry, additives and external electric fields on flame synthesized silica and titania nanoparticles has previously been characterized [135]. Specifically, titania particles synthesized in an inverted diffusion flame were up to 10 times smaller than particles produced in a regular diffusion flame, likely due to altered flame temperature [139]. However, the effect of flame configuration has not been examined for other nanoparticle compositions, specifically iron oxide.
In this study, we initially hypothesized that we can synthesize iron oxide nanoparticles in an easy to scale up and inexpensive method and further control the final nanoparticle morphology and chemistry by changing flame conditions. Iron oxide nanoparticles were synthesized using a coflow diffusion flame configuration. Control over the final iron oxide morphology and chemistry was provided by controlling three parameters: flame configuration, flame temperature and additive loading. The material characteristics of the flame synthesized iron oxide nanoparticles, including morphology, elemental composition, and particle size were analyzed by transmission electron microscopy (TEM), energy dispersive spectroscopy (EDS), and Raman Spectroscopy.

2.2 Experimental Setup

In the current work, a laboratory scale combustion synthesis and sampling system was developed for direct iron oxide nanoparticle synthesis. The combustion synthesis system was composed of three main components: the burner, which was used to create a high temperature synthesis environment; a liquid precursor delivery system; and two particle sampling mechanisms to obtain discrete and bulk material samples via thermophoretic deposition. A schematic of the combustion synthesis system is shown in Figure 1.

A co-flow diffusion flame burner consisting of three concentric stainless steel tubes with 1/8”, 1/2” and 7/8” diameters was used. An inert argon stream carrying the precursor vapor was introduced through the center tube. Oxidizer (oxygen) and methane were passed through the second and third tubes respectively to achieve an inverse
diffusion flame, or in the reverse order for a diffusion flame configuration. Nitrogen was introduced through the methane line to cool the flame temperature. The burner and exhaust were surrounded by a rectangular acrylic chimney to facilitate stable burning and to prevent ambient air entrainment.

The gas phase delivery system for the liquid precursors consisted of a temperature controlled aluminum canister containing the liquid precursor. Argon gas entered the canister and bubbled through the liquid precursor in a convoluted path before exiting to the burner. The gas lines, delivery tube, and burner were heated using heat tape controlled to a required temperature to prevent precursor condensation. Temperature measurements along the delivery system were made with K-type thermocouples and OMEGA CNi3222 temperature controller. Rotameters measured oxygen, methane, argon and nitrogen flow rates. Studies were completed to examine the time required to reach steady state temperatures in the delivery system for different precursor materials.

Two different sampling systems were developed to analyze the synthesized nanoparticles. Bulk nanoparticle samples were collected 10 cm above the flame on a water-cooled cold plate by thermophoretic deposition for sampling times of approximately 10 minutes. Discrete samples were thermophoretically collected onto TEM grids (Electron Microscopy Sciences, carbon film, 300 mesh copper), connected to a timer controlled pneumatic cylinder, which was inserted into the flame at different heights for sampling times of less than one second (~100 ms). This technique was modified and extensively employed by Dobbins and Megaridis for collecting soot from flames to study its morphology [140].
Figure 1. Experimental setup schematic

The discrete samples were studied using TEM (JEM-2000FX) to identify particle morphology (including particle size) and high-resolution TEM (JEM-2010F FasTEM field-emission gun transmission electron microscope) for detailed lattice structure examination. Samples were also examined using TEM-EDS for elemental analysis. The iron oxide nanoparticle elemental composition was analyzed using a Renishaw RM1000 VIS Raman Microspectrometer with 633 nm excitation wavelength. Laser power was kept under 25 percent total power to avoid sample degradation and before and after each spectrum had been recorded. A visual inspection under white light was completed to
detect any compositional changes caused by the laser power.

Four different synthesis conditions were examined in the study: Case I- inverse diffusion flame; Case II- inverse diffusion flame cooled with N₂; Case III- diffusion flame cooled with N₂; and Case IV- inverse diffusion flame with titanium-tetra-isopropoxide (TTIP), Ti[OCH(CH₃)₂]₄, additive loading. Table 1 summarizes the operating conditions. All compressed gases (H₂, O₂, Ar, N₂) were obtained from Airgas with greater than 99.99% purities. Iron pentacarbonyl, Fe(CO)₅, and titanium-tetra-isopropoxide (TTIP), Ti[OCH(CH₃)₂]₄, were used as the liquid precursors (Alfa-Aesar) for the synthesis of iron oxide and titanium oxide, respectively and delivered to the reactor as saturated vapors carried by Argon gas. All synthesis conditions were conducted under oxygen rich conditions with an equivalence ratio of 0.28 to minimize unwanted carbon contamination in synthesized nanoparticles.
Table 1. Synthesis conditions

<table>
<thead>
<tr>
<th>Case</th>
<th>Synthesis Condition</th>
<th>Material System</th>
<th>CH\textsubscript{4} [l/min]</th>
<th>O\textsubscript{2} [l/min]</th>
<th>Ar [l/min]</th>
<th>N\textsubscript{2} [l/min]</th>
<th>Ø</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Inv. Diffusion</td>
<td>Iron Oxide</td>
<td>0.494</td>
<td>3.546</td>
<td>0.305</td>
<td>-</td>
<td>0.28</td>
</tr>
<tr>
<td>2</td>
<td>Inv. Diffusion</td>
<td>Iron Oxide</td>
<td>0.494</td>
<td>3.546</td>
<td>0.305</td>
<td>4.000</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>Diffusion</td>
<td>Iron Oxide</td>
<td>0.494</td>
<td>3.546</td>
<td>0.305</td>
<td>4.000</td>
<td>0.28</td>
</tr>
<tr>
<td>4</td>
<td>Inv. Diffusion</td>
<td>Iron Oxide-Titania</td>
<td>0.494</td>
<td>3.546</td>
<td>0.305</td>
<td>-</td>
<td>0.28</td>
</tr>
</tbody>
</table>

2.3 Results

2.3.1 Case I vs Case II

The effect of flame temperature on iron oxide nanoparticle chemical composition and morphology was examined by comparing an uncooled inverse diffusion flame to an N\textsubscript{2} cooled inverse diffusion flame. CH\textsubscript{4}, O\textsubscript{2}, and Ar gas flow rates were kept constant to isolate the effect of N\textsubscript{2} gas cooling, and a 0.28 equivalence ratio was used to minimize unwanted carbon contamination.

For the Case I conditions given in the Table 1, a 2493K adiabatic flame temperature was achieved as calculated by STANJAN program and JANNAF thermochemical tables. Raman spectroscopy indicated that nanoparticles were a
heterogeneous mixture of two common iron oxide forms: hematite and magnetite (Figure 2). For hematite, the expected seven photon lines in the Raman spectrum of two A1g modes and five Eg modes (225, 498, 247, 293, 299, 412 and 613 cm\(^{-1}\)) were observed [141]. Since hematite is an antiferromagnetic material, the collective spin movement can be excited in a magnon which is also detected by Raman spectroscopy. The intense peak observed at 1320 cm\(^{-1}\) is assigned to two-magnon scattering which arises from interaction of two magnons created on antiparallel close spin sites [141]. For magnetite, disagreements exist in the literature for the Raman peak locations, especially for peaks close to reported hematite peaks. Magnetite has five Raman bands: three T2g, one Eg and one A1g (298, 320, 420, 530 and 680 cm\(^{-1}\)). For our nanoparticles, we observed wide peaks at 320, 530 and 680 cm\(^{-1}\), which are reported in the literature as the characteristic magnetite peaks [141].

In Case II, the system was cooled to 1686K adiabatic flame temperature by flowing 4000 ml/min N\(_2\) through the third annulus along with CH\(_4\). All other conditions were kept constant to obtain the same equivalence ratio of 0.28. Raman spectroscopy of synthesized nanoparticles indicated that the nanoparticles were again a heterogenous mixture of hematite and magnetite (Figure 2). However, in these cooled conditions, almost all hematite peaks were diminished, showing that the final product was primarily composed of magnetite.

TEM analysis was employed to compare iron oxide nanoparticle morphology for the two different synthesis conditions as discussed above. Nanoparticles synthesized under higher temperature conditions, Case I, were mainly monodispersed 6-12 nm iron
oxide nanoparticles and aggregated clusters of these 6-12 nm nanoparticles accompanied with larger 50-60 nm polygonal shaped iron oxide nanoparticles (Figure 3A, B). In contrast, nanoparticles synthesized under the cooler conditions in Case II were mainly 6-12 nm monodispersed iron oxide nanoparticles when they were collected 5mm above the burner exit (Figure 3C) and aggregated clusters of these nanoparticles when they were collected 50 mm above the burner exit (Figure 3D).

Figure 2. Raman spectroscopy showed nanoparticles synthesized in Case I were a heterogeneous mixture of hematite and magnetite. In Case II, N₂ addition to the CH₄ line decreased the flame temperature and hematite peaks, demonstrating that cooler flame temperatures produced mainly magnetite nanoparticles.
Figure 3. Iron oxide nanoparticles synthesized in Case I conditions were aggregated clusters of 6-12 nm nanoparticles and 50-60 nm polygonal nanoparticles (A, B). However iron oxide nanoparticles synthesized in Case III conditions were 6-12 nm monodisperse nanoparticles (C) and aggregated nanoparticle clusters (D).

2.3.2 Case II vs Case III

In this study, the effect of flame configuration on iron oxide nanoparticle chemical composition and morphology was investigated. CH₄, O₂, Ar and N₂ gas flow rates were kept constant, and the locations for fuel and oxidizer injection were
reconfigured. In Case II, O₂ flowed through the second annulus and CH₄ flowed through the third annulus to achieve an inverse diffusion flame configuration. In Case III, CH₄ flowed through the second annulus and O₂ through the third to obtain a diffusion flame configuration.

No significant differences in nanoparticle chemical composition were observed by Raman spectroscopy for the different flame configurations in Case II and Case III (Figure 4). The effect of flame configuration on the nanoparticle morphology was investigated by TEM. Nanoparticles were thermophoretically collected on TEM grids at several flame heights above the burner exit to examine the progress of nanoparticle growth along the flame axis. For both Case II and Case III conditions, thermophoretic sampling and TEM analysis show that only singlet particles exist 5 mm above the burner exit (Figure 5). Iron oxide nanoparticles produced by Case II inverse diffusion flame conditions (Figure 6A) were smaller compared to nanoparticles produced by Case III diffusion flame conditions (Figure 6B). As nanoparticles advanced in the flame, they became more aggregated and an increase in nanoparticle size was observed (samples collected 50 mm above the burner exit) (Figure 6C, D).
Figure 4. Raman spectroscopy showed that there is no significant difference in terms of chemical composition between the particles synthesized using the Case II and Case III conditions.
Figure 5. A TEM image of nanoparticles collected at the burner exit showed highly monodisperse iron oxide nanoparticles 6-12 nm in diameter.
Figure 6. Iron oxide nanoparticles produced with Case II (inverse diffusion flame) conditions (A) were smaller in size to nanoparticles produced with Case III (diffusion flame) conditions (C). As nanoparticles advanced in the flame, they become more aggregated and increased in size (B, D).

2.3.3 Case I vs Case IV

In Case IV, the effect of TTIP additive loading on nanoparticle chemical composition and morphology was examined in the inverse diffusion flame configuration. The bubblers containing the precursors were set at the boiling temperatures of TTIP (BP = 104°C) and Fe(CO)₅ (BP = 103°C) to obtain saturated precursor vapors. The Ar stream carrying the Fe(CO)₅ flowed through the burner center annulus, and the Ar stream
carrying the TTIP flowed through the outer annulus with the CH₄. When the Fe(CO)₅ precursor flow was prevented, the sample Raman spectrum showed the standard Raman peaks for two well known forms of anatase TiO₂ (144, 197, 400, 525, and 650 cm⁻¹), indicating that the nanoparticles were mainly composed of the anatase phase of TiO₂ (Figure 7) [142]. The external morphology of the synthesized anatase nanoparticles is presented in Figure 8A. In the HRTEM image of the synthesized TiO₂ nanoparticles, anatase phase nanocrystallites can be identified from the 0.352 nm lattice fringes, which corresponds to the (1 0 1) lattice plane of the anatase phase TiO₂ (Figure 8B). The lattice plane and crystallinity is further evident in the insert of Figure 8B, which corresponds to the fast Fourier transformation (FFT) power spectrum of the selected area in the HR-TEM image (Figure 8B).

Figure 7. Raman spectrum showed that the nanoparticles were composed of the anatase form of TiO₂.
To investigate the effect of TTIP loading on iron oxide nanoparticle morphology and chemistry, Ar flowrate carrying the TTIP was set to 100ml/min and Ar flowrate carrying the Fe(CO)$_5$ was set to 305ml/min. At this configuration, the synthesized nanoparticles were mainly iron oxide (hematite and magnetite forms). TEM images showed that these iron oxide nanoparticles were highly monodispersed and spherical in shape, with sizes ranging from 50 to 90 nm (Figure 9A). EDS analysis verified the iron content of the particles (Figure 9B). The iron oxide nanoparticles synthesized in Case I and Case IV conditions were compared to determine the effect of TTIP additive on nanoparticle morphology (Figure 10). TTIP loading resulted in synthesis of
monodispersed, large iron oxide nanoparticles. The small aggregated iron oxide particles observed in Case I conditions (Figure 10A, B) were not observed in Case IV conditions.

Figure 9. TEM images of the iron oxide nanoparticles synthesized in Case IV conditions showed highly monodisperse 50-90nm size nanoparticles (A). EDS analysis verified the nanoparticle iron oxide content (B).
Figure 10. TEM analysis verified that nanoparticles synthesized using Case IV (C, D) conditions were morphologically different than nanoparticles synthesized using Case I conditions (A, B). The nanoparticles synthesized with TiO$_2$ doping were more monodisperse with sizes from 50nm-90nm and aggregated small nanoparticles were not observed (C, D).

When the Ar flowrate carrying both the TTIP and Fe(CO)$_5$ precursors was set at 305ml/min, the synthesized nanoparticles were a heterogeneous mixture of anatase and iron oxide (hematite and magnetite forms) particles. EDS analysis verified the Ti and Fe
content of the heterogeneous mixture (Figure 11A). The Raman spectrum also showed the anatase, hematite and magnetite content of the particles (Figure 11B). Carbon peaks in Raman spectrum and EDS analysis showed that 305 ml/min TTIP addition resulted in some unburned fuel and soot formation.

Figure 11. Raman spectrum showed that the synthesized nanoparticles were a heterogeneous mixture of anatase, hematite and magnetite nanoparticles when TTIP and Fe(CO)₅ flowrates were set at 305ml/min (B). EDS analysis also verified Fe, Ti and O content of the synthesized nanoparticles (A).

2.4 Discussion

We now show that hematite αFe₂O₃ and magnetite Fe₃O₄ iron oxide nanoparticles, which are used in diverse applications from biomedical imaging contrast agents to catalysts to gas sensors, can be synthesized using a coflow diffusion flame. The size and
The morphology of synthesized iron oxide nanoparticles was highly dependent on flame temperature, residence time, flame configuration, and additives when CH$_4$, O$_2$, Ar and N$_2$ gas flow rates were kept constant. A cooled inverse diffusion flame produced monodispersed 6-12 nm iron oxide nanoparticles when samples were collected directly above the burner. Thus flame synthesis could produce large quantities of small iron oxide nanoparticles without any required post-processing.

The nanoparticle growth mechanism and overall particle morphology primarily depend on precursor concentration, flame temperature, and particle flame residence time. Additional system parameters, including flame configuration, stoichiometry, gas flow rate, and diluent concentration, are important to the extent that they affect flame temperature and particle residence time [44]. Since precursor concentration and stoichiometry were kept constant in all of our experiments, the morphology of our synthesized iron oxide nanoparticles was a function of flame temperature and residence time. These parameters varied with flame configuration, coolant flow rate, additive loading, and sampling location. Based on our experimental results and careful review of the literature, we propose the following growth mechanism for flame synthesized iron oxide nanoparticles.

The iron pentacarbonyl precursor was exposed to high flame temperatures as it was injected into the burner. At high temperature, iron oxide monomers formed by gas phase chemical reactions. Monomers then polymerized, leading to homogeneous nucleation of 6-12 nm diameter primary particles. Depending on flame temperature and particle flame residence time, these primary particles remained monodispersed, collided
to form branched agglomerates, or coalesced to form larger individual particles [43, 44]. When iron oxide nanoparticles were synthesized in a nitrogen cooled inverse diffusion flame (Case II, 1686K adiabatic flame temperature) and collected 5 mm above the burner exit, the low flame temperature and short residence time resulted in 6-12 nm diameter monodispersed nanoparticles (Figure 3C). Under the same synthesis conditions but at a higher collection point (50 mm above the burner exit), the longer residence time allowed these primary particles to collide and form fractal-like agglomerates consisting of 6-12 nm primary particles (Figure 3D). Agglomerates formed rather than larger nanoparticles because the collision rate was faster than the coalescence rate. However, when iron oxide nanoparticles were synthesized in an inverse diffusion flame without cooling (Case I, 2493K adiabatic flame temperature), the higher flame temperature led to a coalescence rate that was faster than the collision rate [46]. In these conditions, even for short flame residence times, some of the aggregated primary iron oxide nanoparticle clusters coalesced to form larger 50-60 nm spherical and polygonal nanoparticles (Figure 3A, B). Despite the varied larger nanoparticle shapes, they likely formed by coalescence of smaller primary nanoparticles. These larger nanoparticles do not show any agglomeration because any clustered nanoparticles would have coalesced at the high temperature locations where the larger nanoparticles formed.

Diffusion flames generate higher temperatures than inverse diffusion flame configurations, and therefore have been shown to produce larger oxide nanoparticles (SiO₂, SnO₂, TiO₂, Al₂O₃) [139]. By altering the position of fuel and oxidant streams in methane-air diffusion flame reactors, the average primary particle size of TiO₂ powders
made by TiCl₄ oxidation could be changed by as much as a factor of 10. We now show similar results for iron oxide nanoparticles. Even in a nitrogen cooled diffusion flame (Case III), nanoparticles experienced higher flame temperatures compared to the inverse diffusion flame configuration. Case III also produced a narrow flame front, which could have increased the flame temperature. Since the fuel flow rate through the second ring was substantially lower than the oxidizer gas flow rate, the flame was positioned near the center of the second ring. Iron oxide nanoparticles synthesized in the higher temperature diffusion flame configuration coalesced to form larger nanoparticles independent of residence time, since the coalescence rate was higher than the collision rate (Figure 6C, D). The dilution of the Ar stream carrying the Fe(CO)₅ precursor with the adjacent CH₄ stream was not significant since both streams had comparable flow rates of 305 and 494 ml/min, respectively.

The presence of additives or dopants affects particle coalescence rate and subsequent nanoparticle size and morphology. Wu et al. [143] found that addition of K⁺ or Na⁺ during oxide flame synthesis reduced the primary particle size. The particles were coated with a charged layer, which reduced particle collision frequency and resulted in a narrower particle size distribution and smaller primary particles. Fotou et al. [144] found that ferrocene reduced large aggregate formation during silica particle synthesis by SiCl₄ oxidation in a diffusion flame reactor. In our experiments, TTIP was added to the inverse diffusion flame configuration in Case IV to determine the effect of additives on iron oxide nanoparticle morphology. The additive resulted in synthesis of a mixture of titania and 50-90 nm iron oxide nanoparticles. The larger particles were likely synthesized
because the TTIP increased flame temperature or created high temperature gradients, either of which could increase the coalescence rate. Large temperature gradients in a flame can enact strong thermophoretic forces on the newly formed particles. This can alter their residence time at the decisive region where oxidation, nucleation, growth, and coalescence occur, thus affecting particle size and morphology.

Flame configuration and additives can affect nanoparticle properties, in addition to changing their size and morphology. Zachariah and Huzarewicz [39] synthesized submicron YBa$_2$Cu$_3$O$_7$ particles by pyrolysis of the corresponding aqueous nitrate salts in an oxy-hydrogen diffusion flame reactor. They found that making these particles in an over-ventilated coflow diffusion flame resulted in super conducting powders while this was not the case when the particles were made in a premixed flame configuration at the same conditions. Vemury and Pratsinis [145] found that addition of SnCl$_4$ or AlCl$_3$ enhanced the transformation of anatase to rutile and reduced the specific surface area of the product TiO$_2$ made by TiCl$_4$ oxidation in a coflow diffusion flame reactor. In contrast, SiCl$_4$ inhibited the transformation of anatase to rutile and increased the specific surface area of the product powder in agreement with investigations in hot-wall aerosol flow reactors for the effect of silicon [146] and aluminum [147] dopants on titania powders.

In our experiments, only the cooler flame (Case II) changed iron oxide nanoparticle properties by synthesizing less hematite as compared to magnetite. Magnetite converts to hematite at elevated temperatures. Experimental research [148-150] has shown that synthetic magnetite oxidizes first to γ-Fe$_2$O$_3$ (maghemite) and then to α-Fe$_2$O$_3$ (hematite) whereas natural magnetite oxidizes only to γ-Fe$_2$O$_3$, usually at more
elevated temperatures. Schmidt and Vermaas [151] examined the effect of heating on magnetite behavior. They concluded that magnetite goes through two oxidation stages when it is heated in air. The first stage is a surface oxidation to hematite, and the second stage is a complete oxidation also to hematite. Przepiera and Przepiera [152] also showed that precipitated magnetite undergoes the following cycle of thermal transformations in atmospheric air: \[ \text{Fe}_3\text{O}_4 \xrightarrow{O_2,T} \gamma\text{Fe}_2\text{O}_3 \xrightarrow{T} \alpha\text{Fe}_2\text{O}_3. \] These results match with the Raman spectrum analysis of samples produced with Case I and Case II configurations. The Case II configuration with lower flame temperature resulted in magnetite iron oxide nanoparticles. However, some of these nanoparticles transformed into hematite iron oxide nanoparticles at elevated temperatures in Case I conditions.

In all of the experimental configurations investigated in this study \( \text{O}_2 \) was used as the oxidizer rather than air since its oxidizer composition is known to have a significant effect on final nanoparticle morphology. Pratsinis et al. [139] showed that using pure oxygen in a diffusion flame reactor resulted in spherical anatase titania particles. When air was used as the oxidant, the particles were rather aggregates with a significant fraction of rutile. Using oxygen leads to faster fuel consumption, higher temperatures and short flames that accelerate precursor oxidation.

Flame synthesis is an excellent method for the synthesis of single component, high-purity powders of small particle size, high specific surface area and controlled particle size distribution. The major disadvantage of flame synthesis is production of particle aggregates in certain configurations. Our research suggests that flame
configuration, cooling, additive loading, and collection location can control nanoparticle size, morphology, and aggregate formation.

In this study iron oxide nanoparticles were synthesized using a coflow diffusion and inverse diffusion flame configuration. The synthesized nanoparticles were composed of two well known forms of iron oxide: hematite $\alpha$Fe$_2$O$_3$ and magnetite Fe$_3$O$_4$. Nanoparticles synthesized in an inverse diffusion flame configuration were smaller than nanoparticles produced in a diffusion flame configuration. Iron oxide nanoparticles synthesized in an inverse diffusion flame configuration with N$_2$ cooling showed no large iron oxide nanoparticles, indicating that the nanoparticles did not coalesce in the lower temperature flame. Raman spectroscopy showed that the nanoparticles were mainly magnetite without hematite, which had dominated in the uncooled inverse diffusion flame configuration. When particles were synthesized in the presence of TTIP additive, larger monodispersed particles were observed. The results of this study indicate that flame synthesis has the potential to create iron oxide nanoparticles with controlled morphology and chemistry for various applications, including gas sensor technology and biomedical engineering.
CHAPTER III: CELLULAR UPTAKE AND TOXICITY STUDIES OF SYNTHESIZED IRON OXIDE NANOPARTICLES

3.1 Introduction

We synthesized iron oxide nanoparticles using flame synthesis as described in Chapter 2. Our end goal was to use flame synthesized iron oxide nanoparticles in biomedical applications. To do so we needed to verify that combustion synthesized iron oxide nanoparticles were equally non-toxic as commercially available iron oxide nanoparticles. To use the synthesized nanoparticles without negatively affecting human health, we also needed to investigate the major mechanisms that lead to nanoparticle toxicity. This will enable fabrication of nanoparticles with different composition or shape that would suppress these mechanisms.

Superparamagnetic nanoparticle interactions with endothelial cells are critically important, since nanoparticles injected into the bloodstream will directly contact endothelial cells lining the inner blood vessel surface. Nanoparticles will need to pass through the endothelial cells to reach any targeted tissue or cell in the body. The endothelium is also an important target for drug and gene therapy since is important in vascular homeostasis. Endothelial cells may be targeted to enhance angiogenesis in wound healing, to inhibit angiogenesis in tumor growth, or to decrease permeability and inflammation in atherosclerosis [153-155]. Nanoparticles provide a unique opportunity to
focus gene and drug therapy on endothelial cells, in particular by binding to endothelial cell specific markers [156-159].

Little is known about the underlying mechanisms resulting in nanoparticle toxicity after the cellular nanoparticle uptake. Several mechanisms for nanoparticle-induced injury of cells and tissues have been proposed and are supported by limited experimental evidence. The currently best developed hypothesis for nanoparticle toxicity is ROS generation [113]. This is thought to result in protein, DNA and tissue injury. There are several hypotheses explaining how nanoparticles generate the ROS. ROS may be directly generated from free radicals on the particle surface. Transition metal nanoparticles like iron can generate ROS through acting as catalysts in Fenton-type reactions, resulting in hydroxyl radical formation [114]. Or ROS might originate from altered mitochondrial function due to nanoparticles uptake into mitochondria [115], where they produce physical damage and contribute to oxidative stress [116].

Several different materials were investigated in terms of ROS induced toxicity. Human bronchoalveolar carcinoma-derived cells exposed to 70 nm ZnO particles showed increased ROS formation and reduced cell viability in a dose and time dependent manner [160]. TiO$_2$ nanoparticle uptake into human bronchial epithelial cells prompted a concentration-dependent generation of intracellular ROS after 6 hours exposure, and ROS formation was statistically different than control cells at concentrations of 10 μg/cm$^2$ and 50 μg/cm$^2$ [161]. Combustion derived nanoparticles were found to cause inflammation via oxidative stress and activation of redox-sensitive transcription factors [117]. Transition metals also induce ROS, which results in DNA strand breakage [119].
Oil fly ash generated ROS and oxidative stress in the lungs [124]. Apopa et al. showed that uptake of iron oxide nanoparticles with an average diameter of 298 nm in human microvascular endothelial cells increased ROS which enhanced cell permeability. However none of the studies mentioned investigated iron oxide dose dependent ROS formation or ROS formation over time.

After nanoparticle endocytosis, nanoparticles may affect the cell cytoskeleton by forming vacuoles in the cell body which disrupt the cell cytoskeleton. Gupta and Curtis [68] previously showed that cells incubated with plain iron oxide nanoparticles showed cell cytoskeletal disruption. However their results were preliminary, were not investigated in detail, and did not show a relationship among iron oxide nanoparticle concentration, cytoskeleton disruption, and cell toxicity. Furthermore, the effect of nanoparticle-induced ROS generation on actin cytoskeleton disruption has not been investigated.

Actin cytoskeleton disruption and ROS formation were previously hypothesized to work in a coupled manner. Studies in yeast showed that decreased actin turnover and accumulation of large F-actin aggregates trigger an increase cytosolic ROS [162]. As previously mentioned, mitochondria are one of the main sources of ROS formation in cells. The reduction in actin dynamics leads to open voltage-dependent anion channels that reduce mitochondrial membrane potential ($\Delta \Psi_m$) which results in increased ROS release and sensitivity to apoptosis [132]. Reduced mitochondrial activity results in reduced ATP generation, which in turn causes reduced actin dynamics. Mitochondrial dysfunction-related ROS release and actin cytoskeleton disruption act as an
interconnected system, stimulating each other back and forth. Therefore, ROS and actin nanoparticle toxicity should be investigated together.

In this study, we initially hypothesized that combustion synthesized iron oxide nanoparticles were equally compatible to commercially available ones. We further hypothesized that iron oxide nanoparticles induce ROS generation in a dose dependent manner, and this ROS formation leads to actin cytoskeleton disruption. We first investigated the interaction of iron oxide nanoparticle with porcine aortic endothelial cells (PAEC). Combustion synthesized nanoparticles were incubated *in vitro* with PAEC, and cell adhesion, viability, and function were compared to cells incubated with commercially available iron oxide nanoparticles. Then we investigated the dose dependent effect of iron oxide nanoparticle uptake on ROS formation and actin cytoskeleton disruption by confocal microscopy. Transmission electron microscopy (TEM) was used to image nanoparticles inside the PAEC. A live dead assay was used to assess viability of cells loaded with different iron oxide nanoparticle concentrations and cells in which ROS formation was blocked. We now show that flame synthesis can efficiently produce superparamagnetic iron oxide nanoparticles that are non-toxic to endothelial cells, and that increased nanoparticle concentration induces ROS formation which disrupts the actin cytoskeleton.
3.2 Materials and Methods

3.2.1 Cell Culture

Porcine aortic endothelial cells (PAEC), isolated by the collagenase dispersion method, were maintained in low glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin, and 2% glutamine (Invitrogen). Culture medium was changed every 48 hours and cells between passages 4 and 9 were used. Cells were seeded in 12 well plates at confluence in 2 ml complete medium and incubated at 37°C in a 5% CO₂ incubator for 24 hours. For ROS measurement, cells were seeded at confluence in 2 ml complete medium in 35mm glass bottom dishes with a 14mm microwell diameter and 1.5 mm glass thickness for live cell confocal microscopy. Cells were incubated at 37°C in a 5% CO₂ incubator for 24 hours. For each experiment, complete medium was replaced with the same medium supplemented with increasing concentrations of nanoparticles and incubated for an additional 24 hours. Negative control cells were incubated without nanoparticles, and positive control cells were incubated with 10 ng/ml tumor necrosis factor-α (TNFα).

3.2.2 Nanoparticles

Combustion synthesized nanoparticles or NanoArc magnetic iron oxide nanoparticles (Alfa Aesar, Ward Hill, MA) of 20-40 nm diameter were used in all experiments.
3.2.3 Live-Dead Assay for Cell Viability

The Live-Dead assay (Molecular Probes) quantifies alive and dead cells by measuring intracellular esterase activity and plasma membrane integrity. Nonfluorescent dye Calcein AM converts to fluorescent calcein in the presence of intracellular esterase activity, producing a uniform green fluorescence in live cells. Ethidium homodimer-1 enters cells with damaged membranes and binds to nucleic acids, producing red fluorescence in dead cells.

For viability comparison between flame synthesized and commercially available iron oxide nanoparticles PAEC were seeded on sterile 13-mm round glass coverslips and cultured in 12-well tissue culture plates. After 24 hours, culture medium was replaced by medium with increasing nanoparticle concentrations. PAEC were incubated with nanoparticles for 24 hours, washed with phosphate-buffered saline (PBS), and treated with 150 μl solution of 2 μM calcein AM and 4 μM EthD-1 as per manufacturer instructions. Cells were incubated at room temperature for 45 minutes and viewed in an Olympus IX81 inverted fluorescent microscope.

For assessing viability of magnetically labeled cells whose ROS formation was blocked, PAEC were seeded on 35mm glass bottom microwell dishes. After 24 hours, some samples were pre-incubated in 4 mM N-acetyl cysteine (NAC) to scavenge intracellular ROS and 50 mM sodium pyruvate to scavenge extracellular ROS for 2 hours. PAEC were incubated with increasing concentrations of nanoparticles for 24 hours, washed with phosphate-buffered saline (PBS), and treated with a 200 μl solution of 2 μM calcein AM and 4 μM ethidium homodimer-1 as per manufacturer instructions.
Cells were incubated at room temperature for 45 minutes and viewed in an Olympus IX81 inverted fluorescent microscope.

3.2.4 Membrane Integrity Assay

The CytoTox-ONE Homogenous membrane integrity assay kit (Promega, Madison, WI) measures release of lactate dehydrogenase (LDH) from cells with damaged membranes through the conversion of resazurin into fluorescent resorufin. PAEC seeded in 12-well tissue culture plates were incubated with nanoparticles as described previously. After 24 hours, 100 μl of medium from each sample was transferred into a 96 well flat-bottomed black plate. 100 μl of CytoTox-ONE reagent was added to each well, after which the plate was incubated at room temperature for 10 minutes protected from light. Fluorescence was measured at 560/590 nm in a GENios microplate reader.

3.2.5 Alamar Blue Cell Viability Test

Alamar blue quantitatively measures cell proliferation and metabolic activity using an oxidation-reduction (REDOX) indicator that fluoresces and changes color in metabolically active cells. After 24 hours of nanoparticle exposure, 100 μl cell medium was transferred into 96 well flat-bottomed black assay plates. 10 μl Alamar blue solution (AbD Serotec Ltd, Oxford, UK) was added to each well and the well plate was incubated for 4h at 37°C in 5% CO₂ atmosphere. Fluorescence was measured 535/590 nm in a GENios microplate reader.
3.2.6 Cell Proliferative Response to Fibroblast Growth Factor-2

Fibroblast growth factor-2 (FGF-2) is heparin binding growth factor which induces proliferation and enhanced survival in endothelial cells, among other effects. PAEC were seeded in 6-well tissue culture plates. After 24 hours, medium was changed to medium supplemented with 0.1 mg/ml either purchased or synthesized iron oxide nanoparticles. Concurrently, 10 ng/ml FGF-2 was added to cells. Medium was changed and fresh FGF-2 added after 2 days. On days 3 and 5, 200μl Alamar blue (AbD Serotec Ltd, Oxford, UK) was added to each well. After 4 hours, 100 μl of medium, with Alamar blue from each sample was transferred into 96 well flat-bottomed black plates. Fluorescence was measured at 535/590 nm in a GENios microplate reader.

3.2.7 Actin Cytoskeleton Labeling

PAEC incubated with different concentrations of iron oxide nanoparticles were washed with PBS and fixed using 4% paraformaldehyde for 20 minutes. 0.1% v/v Triton X-100 in PBS was added to fixed cells for 5 minutes. After washing with PBS three times for 5 minutes, cells were blocked with 1% BSA in PBS for 30 minutes. To label the actin cytoskeleton, cells were incubated with 200 μL rhodamine phalloidin (1 unit/well) in 1% BSA for 20 minutes. To label the nuclei, cells were incubated with 200 μL Hoechst (bis-benzimide, 1 μg/mL) in 1% BSA for 30 minutes. Cells were washed three times with PBS and stored at 4°C in PBS. Inverted coverslips were mounted on cover slides for confocal microscopy. Cell length was measured by measuring the distance between the
two sharp end points of the elongated cell. Cell length data in each condition is average of five individual cell length measurements.

3.2.8 Reactive Oxygen Species Labeling

Image iT Live Green Reactive Oxygen Species Detection Kit from Invitrogen was used for detection of ROS in live cells. The assay is based on 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), a reliable fluorogenic marker for ROS in live cells. It also uses cell-permeant nucleic acid stain Hoechst 33342 to label cell nuclei. The nonfluorescent carboxy-H2DCFDA permeates live cells and is deacetylated by nonspecific intracellular esterases. In the presence of nonspecific ROS produced throughout the cell, particularly during oxidative stress, the reduced fluorescein compound is oxidized and emits bright green fluorescence. Cells seeded on 35 mm glass bottom microwell dishes and incubated with different concentrations of iron oxide nanoparticles were labeled for ROS according to manufacturer’s protocol. Live cell imaging using an Olympus IX81 confocal microscope was performed with cells in HEPES buffer. Fluorescence intensity was measured with an emission wavelength of 495 nm and excitation wavelength of 529 nm for Carboxy-H2DCFDA and with an emission wavelength of 350 nm and excitation wavelength of 461 nm for Hoechst.

3.2.9 Scanning Electron Microscopy for Cell Morphology

Scanning electron microscopy (SEM) was used to visualize nanoparticle interaction with cells. PAEC were seeded on 8 mm sterile glass coverslips in 12 well
plates and loaded with increasing nanoparticle concentrations as previously described. Cells were fixed in 3% glutaraldehyde (HMDS; EMS, Hatfield, PA) at room temperature for 20 minutes, rinsed three times with PBS for 5 minutes each, and dehydrated in graded ethanol (Pharmco, Brootfield, CT). Ethanol was then replaced with hexamethyldisilazane (EMS, Hatfield, PA), and samples were dried by overnight HMDS evaporation in a chemical fume hood. Samples were desiccated under vacuum for two days, sputter coated with 0.75 nm thick Pt/Pd layer to increase conductivity, and viewed under a Zeiss Supra 50VP SEM.

3.2.10 Transmission Electron Microscopy

Samples for transmission electron microscopy (TEM) were fixed with 4% paraformaldehyde overnight at 4°C. The cells were washed with 0.1M cacodylate buffer prior to post fixation in 2% osmium tetroxide at room temperature. After several additional buffer washes, the samples were dehydrated in a graded ethanol series prior to embedding in PolyBed 812 (Polysciences, Warrington, PA). The cells were then sectioned en face, stained with uranyl acetate and bismuth subnitrite, and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capturing software.
3.2.11 Statistical Analysis

Samples were statistically compared using Student’s t-test. Statistical significance was established at either p<0.05 (#) or p<0.01 (*). Two-way ANOVA was used to compare changes over time, with statistical significance established at p<0.0001.

3.3 Results

3.3.1 Nanoparticle Synthesis and Analysis

Two different size modes of iron oxide nanoparticles were synthesized using the flame synthesis method previously described in Chapter II. Iron pentacarbonyl, Fe(CO)$_5$, was used as the liquid precursor and delivered to the reactor as saturated vapor carried by argon gas. All synthesis conditions were conducted under oxygen rich conditions with low equivalence ratios to minimize unwanted carbon contamination in synthesized nanoparticles. Inverse diffusion flame configuration, with oxidizer in the second annulus and fuel in the third annulus, resulted in a high concentration of magnetite formation as verified by Raman spectroscopy. For this reason, all iron oxide nanoparticles in this chapter were synthesized using the inverse diffusion flame configuration. A table of operating conditions can be found in Table 2.
Table 2. The operating conditions were arranged to have an inverse diffusion flame with oxygen rich conditions in order to prevent unwanted carbon contamination and produce magnetite rich iron oxide nanoparticles.

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<tr>
<td>Fe(CO)₅</td>
<td>494</td>
<td>3546</td>
<td>305</td>
<td>4000</td>
<td>0.28</td>
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Representative TEM images of flame synthesized iron oxide nanoparticles collected at 5 cm above the burner exit are shown in Figure 12. Monodispersed iron oxide nanoparticles 6 – 12 nm in diameter (Figure 12A, B) were synthesized. In some cases, nanoparticle clusters formed due to excessive heat (Figure 12C). Significant Fe Kα, Fe Kβ, Fe Lα and O peaks were observed in the EDS spectrum (Figure 12D), confirming the iron and oxygen content of the nanoparticles. Since no significant C peak was observed, the high oxygen concentration of the flame successfully prevented unwanted carbon contamination. The copper Cu Kα and Cu Kβ peaks in EDS spectrum were background from the TEM grids.
Figure 12. TEM images of the flame synthesized iron oxide nanoparticles collected at 5 cm above the burner exit showed that monodispersed iron oxide nanoparticles 6 – 12 nm in diameter were synthesized (A, B). In some cases, nanoparticle clusters formed due to excessive heat (C). EDS analysis results of flame synthesized iron oxide nanoparticles verified the Fe and O composition of the nanoparticles with no carbon contamination (D).

Raman spectroscopy of synthesized nanoparticles (Figure 13) indicated that the nanoparticles were a heterogenous mixture of two common forms of iron oxide, hematite and magnetite. For hematite, seven photon lines are expected in the Raman spectrum of two $A_{1g}$ modes and five $E_g$ modes (225, 498, 247, 293, 299, 412 and 613 cm$^{-1}$) [141]. Hematite is an antiferromagnetic material and the collective spin movement can be
excited in a magnon. The intense peak observed at 1320 cm$^{-1}$ is assigned to a two-magnon scattering which arises from interaction of two magnons created on antiparallel close spin sites [141]. For magnetite there are some disagreements in the literature for the locations of Raman peaks, especially for peaks very close to reported hematite peaks. Magnetite has five raman bands: three T$_{2g}$, one E$_g$ and one A$_{1g}$ (298, 320, 420, 530 and 680 cm$^{-1}$). But wide peaks observed at 320, 530 and 680 cm$^{-1}$ are reported as the characteristic magnetite peaks in published studies [141]. The location of the raman peaks in literature [141] were also indicated in Figure 13 for reference.

Figure 13. Raman Spectrum of the flame synthesized iron oxide nanoparticles showed that nanoparticles synthesized were composed of two well known forms of iron oxide: magnetite, Fe$_3$O$_4$, and hematite, $\alpha$Fe$_2$O$_3$. The location of the raman peaks in literature [141] were also indicated for reference.
3.3.2 Nanoparticle Cellular Uptake

Endothelial cells uptake flame synthesized superparamagnetic nanoparticles in a manner similar to commercially available nanoparticles. PAEC incubated with 0.1 mg/ml both commercially available and flame synthesized iron oxide nanoparticles uptake the nanoparticles inside (Figure 14A, C). No nanoparticles were observed in the cell nucleus and they were all located in the cell cytoplasm (Figure 14B, D).

Scanning electron microscopy images of cell populations incubated with increasing concentrations of nanoparticles are shown in Figure 15. Endothelial cells remained attached and spread after 24 hours of exposure to 0.5 mg/ml nanoparticle solution. While no change in cell adhesion and total attached cell number was observed (Figure 15C, D, E, F, G, H), cell morphology did change with nanoparticle exposure. When incubated with nanoparticles (Figure 16C, D, E, F, G, H), endothelial cells were less spread, more rounded, and appeared to have lost their typical cobblestone morphology.
Figure 14. TEM images of the individual PAEC loaded with 0.1 mg/ml (A) commercially available and (C) combustion synthesized iron oxide nanoparticles showed that iron oxide nanoparticles were taken inside the cells. Both (B) commercially available and (D) combustion synthesized nanoparticles were not uptaken by cell nucleus and were located inside the cell cytoplasm as aggregated nanoparticle clusters.
Figure 15. SEM images of the cells loaded with different concentrations of purchased and synthesized iron oxide nanoparticles showed no significant difference in attached cell number and adhesion (C, D, E, F, G, H), however cell morphology did change with nanoparticle exposure.
Figure 16. Higher magnification SEM images of the individual cells loaded with different concentrations of purchased and synthesized iron oxide nanoparticles showed that when incubated with nanoparticles (C, D, E, F, G, H), endothelial cells were less spread, more rounded, and appeared to have lost their typical cobblestone morphology.
3.3.3 Nanoparticle Cytotoxicity

Flame synthesized superparamagnetic nanoparticles demonstrate low cytotoxicity comparable to commercially available nanoparticles. Endothelial cell viability, as measured by the cell metabolic indicator Alamar blue, did not change with increasing nanoparticle concentration up to 0.1 mg/ml of iron oxide concentration. However at higher nanoparticle concentrations (0.5 mg/ml and 1.0 mg/ml), a decrease in cell metabolic activities was observed (Figure 17A). There was no statistically significant difference in cell viability with flame synthesized vs. commercially available nanoparticles. Endothelial cell membrane integrity, as measured by LDH release, demonstrated a slight loss of membrane integrity with purchased nanoparticles (Figure 17B). However, no statistically significant changes in cell membrane integrity were observed in cells incubated with synthesized nanoparticles even at the highest iron oxide concentrations.
Figure 17. (A) Endothelial cell viability, as measured by the cell metabolic indicator Alamar blue, did not change with increasing nanoparticle concentration up to 0.1 mg/ml of iron oxide concentration. However at higher nanoparticle concentrations (0.5 mg/ml and 1.0 mg/ml), a decrease in cell metabolic activities was observed (A). Endothelial cell membrane integrity, as measured by LDH release, demonstrated a slight loss of membrane integrity with purchased nanoparticles (B). There was no statistically significant difference in cell viability and membrane integrity of the cells loaded with flame synthesized vs. commercially available nanoparticles. (▱ P<0.05 and * P<0.01 relative to control cells)
Endothelial cell viability with both synthesized and purchased nanoparticles was confirmed using a Live/Dead cell viability assay. Fluorescent images of representative cell regions showed no observable difference in live and dead cell density (Figure 18B). When dead cell number was quantified using a microplate reader, samples loaded with high concentrations of both purchased and synthesized iron oxide nanoparticles showed slightly increased cell death over control cells (Figure 18A). However, there was no statistically significant change in cell death for the two types of nanoparticles.
Figure 18. Dead cell number quantified using a microplate reader showed slightly increased cell death in cells loaded with purchased and synthesized iron oxide nanoparticles over control cells (A). No statistical difference between the toxicity levels of PAEC loaded with different concentrations of commercially available and flame synthesized iron oxide nanoparticles was observed.
3.3.4 Endothelial Cell Proliferation with Internalized Nanoparticles

PAEC nanoparticle exposure did not affect cell proliferation induced by FGF-2. PAEC with 0.1 mg/ml of either combustion synthesized or purchased nanoparticles showed increased proliferation by Alamar blue on the fifth day following cell seeding (Figure 19A). No statistically significant difference was observed in cell proliferation between cells exposed to combustion synthesized nanoparticles or commercially available nanoparticles. Phase contrast images confirmed that cells exposed to nanoparticles respond to FGF-2 in a similar manner to control cells in terms of cell morphology. While cells not stimulated by FGF-2 exhibited a typical cobblestone architecture, cells stimulated with 10 ng/ml FGF-2 became elongated and formed swirling patterns independent of nanoparticle concentration or source (Figure 19B).
Figure 19. PAEC loaded with 0.1 mg/ml of synthesized and purchased iron oxide nanoparticles showed an increase in cell proliferation in the presence of FGF-2 by Alamar blue assay (A), and responded to FGF-2 by becoming elongated and forming swirling patterns (B). (* P<0.01 relative to w/o FGF-2 condition)
3.3.5 Effect of Nanoparticle Concentration on ROS Formation

Nanoparticles inside PAEC increased intracellular ROS formation over time. Cells loaded with 0.5 mg/ml iron oxide nanoparticles started to show an increased ROS after 1 hour. We observed a 480% increase in ROS formation at 2 hours, and an 820% increase in ROS formation at 3 hours (Figure 20A). No additional increase in ROS formation was observed after 4 hours. Confocal images show increase in green fluorescence signal intensity from Carboxy-H$_2$DCFDA (Figure 20C). ROS formation also increased with increasing iron oxide concentration. PAEC loaded with 0.1, 0.5 and 1.0 mg/ml iron oxide nanoparticles had 100%, 250% and 350% more ROS inside the cell cytoplasm, respectively (Figure 20B).
Figure 20. Nanoparticles increased cellular ROS formation. (A) ROS formation increases with time of nanoparticle exposure. (B) ROS formation increased with increasing iron oxide concentration. (C) Intensity of the green fluorescence signal from Carboxy-H$_2$DCFDA increases up to 3 hours. Scale bar is 100µm.
3.3.6 Actin Cytoskeleton Elongation with Increasing Nanoparticle Concentration and Over Time

Actin cytoskeleton disruption was observed when cells were loaded with 0.01, 0.05, 0.1, 0.5 and 1.0 mg/ml iron oxide nanoparticles (Figure 21A). As nanoparticle concentration increased, cells and actin stress fibers elongated. Cells loaded with 0.5 mg/ml iron oxide nanoparticles elongated approximately 140% and cells loaded with 1.0 mg/ml iron oxide nanoparticles elongated 153%. The average cell length increased from 32 µm for control cells to approximately 77 µm and 81 µm for cells loaded with 0.5 mg/ml and 1.0 mg/ml iron oxide nanoparticles, respectively. Confocal images show the increase in cell length with increasing nanoparticle concentration (Figure 21C). Actin fiber elongation also increased over time. PAEC loaded with 0.5 mg/ml iron oxide nanoparticles did not show an increase in actin length for the first 4 hours. Average actin fiber length increased 44%, 88%, and 122% at 6, 8, and 12 hours respectively compared to control cells (Figure 21B). After 12 hours, no additional increase in actin length was observed.
Figure 21. Nanoparticles disrupted the actin cytoskeleton. (A) Cell length increased when PAEC were loaded with iron oxide nanoparticles. (B) Actin cytoskeleton elongation occurred between 4 and 12 hours. (C) Confocal images show the cell and actin elongation. Cells lost their cobblestone morphology and become elongated when loaded with iron oxide nanoparticles. (n=3, # p < 0.05 relative to ctrl sample)
3.3.7 Effect of ROS Blockers on ROS Formation and Actin Cytoskeleton Elongation

PAEC were loaded with 0.5 mg/ml iron oxide nanoparticles and ROS production was prevented by adding 4 mM N-acetyl cysteine (NAC) and 50 mM sodium pyruvate. For samples where ROS formation was blocked, no change in actin length was observed. This suggests that blocking ROS production blocks actin cytoskeleton disruption. When ROS formation was induced by tert-butyl hydroperoxide (TBHP) as a positive control, a 45% increase in actin length was observed with respect to control cells (Figure 22A). NAC and sodium pyruvate did effectively reduce ROS formation (Figure 22B). A 32% and 35% decrease in ROS formation was observed in cells loaded with 1 mg/ml iron oxide nanoparticles when NAC or sodium pyruvate were added (Figure 22B). Confocal images also showed that PAEC loaded with nanoparticles became less spread and elongated, while magnetically labeled cells whose ROS formation was blocked kept their original cobblestone morphology (Figure 22C).
Figure 22. NAC and sodium pyruvate blocked ROS formation, which blocked actin disruption. (A) Actin fibers did not elongate in cells loaded with 0.5 mg/ml nanoparticles when ROS formation was blocked with NAC and sodium pyruvate. (B) In PAEC loaded with 1 mg/ml iron oxide nanoparticles, NAC and sodium pyruvate decreased ROS formation. (C) Confocal images showed that while PAEC loaded with nanoparticles elongated, magnetically labeled cells whose ROS formation was blocked kept their original cobblestone morphology. (n=3, *p < 0.05, relative to ctrl sample)
3.3.8 ROS Blockers Effect on Cell Viability

A Live/Dead assay showed that ROS blockers NAC and sodium pyruvate decreased nanoparticle-induced cell death. 0, 0.1 and 0.5 mg/ml iron oxide nanoparticles did not significantly change cell viability (Figure 23A). However in PAEC loaded with 1.0 mg/ml iron oxide nanoparticles, NAC and sodium pyruvate blocked 66% and 50% of the cell death respectively. Fluorescent images represent live and dead cells labeled with Live/Dead assay dyes (Figure 23B).
Figure 23. ROS blockers NAC and sodium pyruvate reduced cell death due to 1.0 mg/ml iron oxide nanoparticles. (A) PAEC loaded with 1.0 mg/ml iron oxide nanoparticles showed increased cell death, which was reduced by NAC and sodium pyruvate. (B) Fluorescent images representing the live (green) and dead (red) cells in each condition. Scale bar is 100µm.
3.4 Discussion

We demonstrated that combustion synthesis can produce superparamagnetic iron oxide nanoparticles 6 – 12 nm in diameter, and that these nanoparticles are equally biocompatible as commercially available nanoparticles. Combustion synthesis provides significant advantages over other material synthesis processes, including controllable particle size, size distribution, phase and composition. This method is further capable of commercial production rates with minimal post-processing of the final product materials. We further showed that uptake of iron oxide nanoparticles in PAEC results in a dose dependent increase in ROS formation in cells which also induces actin cytoskeleton disruption. The investigation of underlying mechanisms that result in nanoparticle toxicity in cells is crucial since if one could block the mechanisms which result in cellular toxicity, it might be possible to use nanoparticles in various biomedical applications without further worrying about the toxicity issues.

The experimental conditions for iron oxide nanoparticle combustion synthesis were selected to obtain small size nanoparticles with high magnetite content. An inverse diffusion flame configuration, where oxidizer flows through the second annulus and fuel flows through the third annulus, was selected over a diffusion flame configuration, where fuel flows through the second annulus and oxidizer flows through the third annulus. In the diffusion flame configuration, the precursor first reacts with the fuel, creating a flame at the center of the second ring. This process generates larger particles, as the concentrated Fe(CO)$_5$ precursor experiences higher temperatures. In the inverse diffusion flame configuration, the precursor is diluted by the oxidizer before reaching the fuel,
resulting in smaller particle sizes compared to the diffusion flame configuration. This phenomenon has previously been described by Pratsinis et al. [139] for TiO₂ formation at different gas mixing conditions.

A common challenge in combustion synthesis is carbon contamination. In this study, carbon contamination was reduced by producing nanoparticles in oxygen rich conditions. An equivalence ratio (ratio of the actual fuel/air ratio to the stoichiometric fuel/air ratio) of 0.28, prevented carbon contamination, as seen from the lack of carbon peak in EDS studies (Figure 12D). However, the oxygen rich environment raised flame temperature, which would have increased nanoparticle size and decreased magnetite content. This was prevented by flowing an N₂ stream through the third annulus along with CH₄ to cool down the flame temperature.

Nanoparticle size is critical to magnetic properties. Magnetite particles less than 20 nm in diameter exhibit superparamagnetism [163]. Despite the increase in superparamagnetism with decreased nanoparticle size, nanoparticles with diameters less than 10 nm have lower saturation magnetization values [164, 165]. For a superparamagnetic particle with magnetic moment \( m = (\chi \beta / \mu_0) V \), the minimum particle size that can be attracted to field maxima is \( V = 3/2 kT_\beta \mu_0 / \beta^2 \), where \( V, \beta, k, \chi, \mu_0, T \) are the particle volume, magnetic field, Boltzmann constant, magnetic susceptibility, free space permeability, and absolute temperature (Kelvin), respectively [166]. In other words, to move a smaller particle, a higher magnetic field must be provided.

Nanoparticle size is also critical to body tissue distribution. Nanoparticles smaller than 100 nm are internalized in cells, but contrary to intuition, smaller size nanoparticles
do not necessarily have better cellular uptake. For instance HeLa cells show a tendency to uptake 50 nm gold particles over any other size [167], and MCF-7 breast cancer cells uptake 100 nm gold nanoparticles most efficiently [168]. Nanoparticles of less than 10 nm diameter are removed from blood circulation through renal clearance and extravasation [4]. On the other hand, particles with more than 200 nm in size have low blood circulation times because they are isolated by the spleen and removed by phagocytosis [4].

We produced 6 – 12 nm diameter monodispersed nanoparticles, as well as nanoparticle aggregates of less than 100 nm in size (Figure 12). When nanoparticles were sampled at the burner exit, they were monodispersed. As the nanoparticles advanced in the flame, they began to form aggregates due to the high heat [44]. Sonication can break up these aggregates [169], however synthesis of aggregates of individual 6 – 12 nm superparamagnetic nanoparticles with total cluster sizes of less than 100 nm might make it easier to attract and move these nanoparticles under a magnetic field while still keeping them in the blood circulation.

Endothelial cells loaded with nanoparticles attached, adhered and spread on surfaces, which is critical to function of these attachment-dependent cells [170, 171]. It has been previously verified by TEM analysis that iron oxide nanoparticles are internalized by human fibroblast cells [171] and mouse breast cancer cells [69]. Our TEM images also showed that iron oxide nanoparticles were internalized in PAEC and formed nanoparticle aggregates in cell cytoplasm. We showed similar representation of internalized nanoparticles in our SEM images of PAEC loaded with purchased and flame
synthesized iron oxide nanoparticles, and we verified that the observed cell protrusions were iron oxide by EDS. While purchased and synthesized nanoparticles did not alter cell viability at low iron oxide nanoparticle concentrations, cell attachment profile and morphology began to change at higher iron oxide concentrations (0.5 mg/ml). It is likely that these endothelial cells would die and detach if incubated with high nanoparticle concentrations for longer periods of time.

In the present study, the toxic effect of a wide range of nanoparticle concentrations was investigated. Since the nanoparticle concentrations to which endothelial cells are exposed in vivo is yet to be determined, we believe this data will elucidate toxicity-concentration relationships for a wide variety of applications. Our toxicity tests revealed that iron oxide nanoparticles show little cytotoxicity up to a concentration of 0.1 mg/ml. Most importantly, there was no significant difference between in cytotoxicity of cells loaded with commercially available nanoparticles and combustion synthesized nanoparticles. A slight increase in cell death and membrane damage was observed at the higher nanoparticle concentrations of 0.5 mg/ml and 1.0 mg/ml by Live/Dead and LDH assays.

However a significant decrease in Alamar blue fluorescence was observed at nanoparticle concentrations of 0.5 mg/ml and 1.0 mg/ml. Two reasons might account for the difference between the Alamar blue assay and the Live/Dead and LDH assays. First, nanoparticles in the medium might interfere with Alamar blue fluorescence. Upon investigation, we found nanoparticles did affect Alamar blue fluorescence, especially at higher nanoparticle concentrations, but the effect did not alter the overall trend. We
compensated for the nanoparticle effect by normalizing Alamar blue fluorescence with controls consisting of medium with nanoparticles not exposed to cells. In general, the Alamar blue assay data should be compensated for nanoparticles in the medium and confirmed through other toxicity and metabolic activity tests.

Even controlling for the effect of nanoparticles on Alamar blue fluorescence, the fluorescence still decreased as nanoparticle concentration increased. Alamar blue measures cell metabolic activity, which often correlates with cell viability. It is possible that endothelial cells exposed to high nanoparticle concentrations (0.5 mg/ml and 1.0 mg/ml) have a lower metabolic rate, even if cell death does not occur. We hypothesize that the increase in nanoparticle concentration, both purchased and synthesized, slightly increased cell death, however it did decrease cell metabolic activities significantly at high iron oxide nanoparticle concentrations.

Endothelial cell functions beyond survival and metabolic activity are critical for nanoparticles that are functionalized to promote or inhibit a specific cellular process. Nanoparticles could be used to either enhance or inhibit endothelial-cell dependent angiogenesis, the growth of new blood vessels from existing vessels. Stimulating angiogenesis could aid in tissue engineering and wound repair, whereas inhibiting angiogenesis could treat diseases such as arthritis, diabetes and cancer [172]. Nanoparticles are extensively used in tumor therapy since tumors have a selective and faster nanoparticle uptake than healthy tissue [173]. Under an oscillating magnetic field, magnetic nanoparticles inside the tumor cells heat up to 43-45°C and destroy cancer cells
[82-85]. But there is no data in the literature showing the effect of nanoparticle presence inside the healthy or malignant cells on cell proliferation.

FGF-2 is a potent growth, survival and differentiation factor that also plays a critical role in guiding endothelial cells in the angiogenic process [174, 175]. We now show that nanoparticles do not affect cell proliferation in response to a growth factor. Endothelial cells loaded with a low concentration of either synthesized or purchased nanoparticles and stimulated by FGF-2 proliferated in similar manner to control cells. These findings showed that the two types of nanoparticles are not only not toxic to the PAEC up to a certain concentration, but also they do not change the reaction of the cells to certain signaling molecules and stimuli like growth factors. These data demonstrate the feasibility of using magnetic nanoparticles to induce cell proliferation.

Nanoparticles may interfere with biological functions or induce cytotoxicity in several different ways. The chemical composition of some nanoparticles can be toxic. For instance, silver nanoparticles were shown to be more toxic than other metal oxide nanoparticles in rat liver cells due to hypothesized reactive oxygen species generation [176]. The nanoparticle chemical and physical properties, such as particle size and surface coating material, might change the cellular response to nanomaterial uptake. Gupta and Gupta [4] reported that modifying the surface of superparamagnetic iron oxide nanoparticles with pullulan, a nonionic polysaccharide, reduced nanoparticle cytotoxicity in human fibroblasts. In addition, there might be effects caused by the nanoparticle shape and the interaction manner between cells and particles themselves. For instance, it has been reported that carbon nanotubes can pierce cells like needles [177].
We showed that nanoparticle localization in the cell cytoplasm resulted in defects and disruptions in the cellular cytoskeleton, primarily actin fibers. The actin cytoskeleton is a highly dynamic network composed of actin polymers and a variety of associated proteins that mediate essential biological functions in cells, including intra and extracellular movement and structural support. The actin cytoskeleton rapidly changes shape and organization in response to stimuli and cell cycle progression. Therefore orientational distribution of actin filaments within a cell is an important determinant of cellular shape and motility. The cytoskeleton is sensitive to ROS formation and oxidative stress due to the presence of thiol groups on the actin microfilaments [178]. On oxidation, these filaments cross-link, leading to reduced cell motility. In this study we showed that there was an internalization and dose-dependent increase in actin cytoskeletal stress fiber formation following treatment of PAEC with superparamagnetic nanoparticles.

Small particle volume and large surface area makes nanoparticles unique from their bulk counterparts, which may result in direct interaction of atoms and molecules on the particle surface with cellular organelles [113]. A major mechanism for nanoparticle toxic effects is related to nanoparticle-induced oxidant stress response [179]. While at low levels, ROS are involved in regulating normal cell functions, at higher abnormal levels, ROS result in cell injury and death [180]. In this study, it was found that the exposure to iron nanoparticles induces the ROS production in PAEC. Furthermore, we found that ROS levels increase with nanoparticle concentration. We observed an increase in ROS formation up to an iron oxide concentration of 1.0 mg/ml. When we further increased the iron oxide concentration to 2.0 mg/ml and 3.0 mg/ml, we did not observe a further
increase in ROS (data not shown). We believe that this is because cells are saturated with nanoparticles after 1.0 mg/ml and do not take any additional nanoparticles in. High iron oxide nanoparticle concentrations also cover most of the cell surface and blocks the fluorescence generated in the cells due to the ROS formation.

We also showed that cellular ROS level increased in the first 3 hours after nanoparticle exposure, but no additional increase was observed after 4 hours. This suggests that the main nanoparticle uptake occurs in the first 3 hours and then cells saturate and do not take up any more nanoparticles. Data in literature also verifies this nanoparticle saturation effect. HeLa cells and mouse macrophages took up ionic maghemite nanoparticles for the first 3 hours, and then nanoparticle uptake saturated [58].

NAC and sodium pyruvate are intracellular and extracellular ROS scavengers respectively. We observed a decrease in ROS formation in magnetically labeled cells whose ROS production was blocked. Our initial hypothesis was that nanoparticle toxicity was related to ROS formation inside cells. From our previous studies, 1.0 mg/ml iron oxide nanoparticles resulted in a decrease in cell metabolic activities and viability [181]. If the scavengers blocked ROS formation, we expected to observe a decrease in dead cell number in cells loaded with 1.0 mg/ml iron oxide nanoparticles. Our studies showed that ROS scavengers successfully decreased cell death with 1.0 mg/ml iron oxide nanoparticles, verifying that ROS are a major mechanism of nanoparticle toxicity.

Actin cytoskeleton disruption occurred between 4-12 hours after nanoparticle loading, while ROS formation occurred 1 hour after nanoparticle loading. This suggests
that ROS formation first occurs in the cell, and then it mediates actin cytoskeleton disruption. If ROS formation is prevented, a decrease in actin cytoskeleton disruption is expected. When ROS formation was blocked, PAEC labeled with nanoparticles still showed disrupted actin cytoskeleton but the disruption was much smaller than samples without blocked ROS. This suggests that ROS formation and actin cytoskeleton disruption works in a coupled manner, with ROS formation triggering actin cytoskeleton disruption.

In this study, nanoparticle cytotoxicity was assessed by incubating combustion synthesized and commercially available iron oxide nanoparticles with endothelial cells. Cells exposed to both types of nanoparticles attached to surfaces, remained viable and maintained membrane integrity up to 0.1 mg/ml of nanoparticle concentration. However, cell metabolic activity decreased significantly at high iron oxide nanoparticle concentrations. In all cytotoxicity assays, no significant toxicity difference was observed between combustion synthesized and commercially available nanoparticles. Neither nanoparticle type affected cell proliferation induced by FGF-2, as long as the nanoparticle concentration was low.

These data suggest that combustion synthesized iron oxide nanoparticles are comparable to commercially available nanoparticles for biological applications. Combustion synthesis is a relatively simple synthesis process with higher purity products and lower time and energy manufacturing costs. It also shows that when iron oxide nanoparticles were uptaken by PAEC cells they localized inside the cell cytoplasm as aggregated clusters. Nanoparticle uptake increased ROS formation in cells during the first
three hours after the uptake. ROS formation induced actin cytoskeleton disruption, and actin fibers started to elongate after addition of ROS blockers to the PAEC also blocked the actin cytoskeleton elongation, pointing to a coupled reaction between ROS formation and actin cytoskeleton disruption. Future work will include coating and functionalizing nanoparticle surfaces for biological applications, including specific cell targeting and bioactive factor delivery and investigation of the ROS formation and actin cytoskeleton disruption under shear stress conditions.
CHAPTER IV: BIOPRINTED NANOPARTICLES FOR TISSUE ENGINEERING APPLICATIONS

4.1 Introduction

Tissue engineering is an interdisciplinary field that uses engineering and life science principles to advance our knowledge of tissue growth, which is then applied toward the development of biological tissue substitutes to restore organ function [96]. Tissue engineering may require precise patterning of cells and bioactive components to recreate the complex, three-dimensional architecture of native tissue. These cells and bioactive factors may then need to be repositioned during tissue growth in vitro or after implantation in vivo to achieve the desired tissue properties. Certain biological components that are valuable in tissue development, such as stem cells or growth factors or nanoparticles themselves, may induce unwanted effects when the tissue engineered construct is implanted. It may be beneficial to remove these cells and bioactive components entirely prior to implantation for biosafety concerns. Furthermore, it is difficult to noninvasively image and track cells and bioactive factors once they are incorporated into the tissue engineered construct, much less when they are implanted in vivo. Visualization of how the tissue components move and interact is critical to improving our understanding of tissue development.

Many biofabrication techniques have been developed to incorporate living cells into functionalized scaffolds in a reproducible, three-dimensional pattern [98, 99]. Rapid prototyping [102, 103], inkjet-based cell printing [104-106], and microcontact printing
are among the commonly used cell deposition systems for tissue engineering applications. These biofabrication methods allow initial deposition of scaffold and cells in a pre-defined pattern. However, the methods are often expensive, time consuming, require chemically modified surfaces, or cause cell damage due to high temperatures and pressures used in the deposition process. We developed a direct cell writing system for the freeform construction of biopolymer-based three-dimensional tissue scaffolds and cell-embedded tissue constructs [109]. The direct cell writing system uses micronozzles driven by pneumatic microvalves to deposit living cells, scaffold material, and bioactive components such as growth factors in controlled amounts with precise spatial positioning. The system requires no pre-processing, is computer controlled to rapidly produce sample replicates, and operates at room temperature and low pressure to maximize cell viability.

Recently, several new approaches have been proposed to actively pattern cell constructs using external forces, including dielectrophoresis [182], an optical trap [183], or superparamagnetic nanoparticles in a magnetic field [184, 185]. Superparamagnetic iron oxide nanoparticles have been of primary interest for both \textit{in vivo} and \textit{in vitro} applications because they exhibit magnetic behavior only in the presence of a magnetic field [4]. These nanoparticles can be conjugated with proteins or loaded inside cells, are relatively non-toxic, and can be imaged by magnetic resonance imaging (MRI) or computed tomography (CT). \textit{In vivo}, superparamagnetic nanoparticles have been used to target drugs to a treatment site to increase drug efficiency and reduce systemic effects [56]; to enhance gene delivery to target cells since nanoparticles easily cross cell membranes [90, 91]; and to detect vascular tissues such as tumors, since iron oxide
nanoparticles appear dark on MRI images [77, 78]. In vitro, superparamagnetic nanoparticles have been used to create high resolution, two-dimensional cell patterns on non-functionalized surfaces [184]. More recently, Frasca et al. used magnetic fields and magnetic field gradients to achieve three dimensional cell patterning [185]. However the ability of this technique to create complex three-dimensional shapes is highly limited since the only method of shape control is with a magnetic field gradient from magnets placed under the scaffold material.

We combined the initial patterning capabilities of the direct cell writing system with the active patterning capabilities of superparamagnetic nanoparticles. This new hybrid technique allows biofabrication of a complex three-dimensional tissue scaffold of magnetically labeled cells and bioactive factors, which could then be manipulated and tracked within the tissue engineering construct. We further assessed the role of the biofabrication conditions in nano-bioprinting system efficacy. Specifically, we investigated the effect of printing parameters and scaffold biopolymer properties on cell viability, nanoparticle manipulation, and patterning capabilities.

Two primary printing parameters in cell writing systems are nozzle diameter and printing pressure. During printing, cells are exposed to shear stress several orders of magnitude higher than shear stress experienced in vivo [186]. These mechanical forces may alter cell function, and in extreme cases, compromise cell viability [187]. For direct cell writing systems, both decreasing nozzle diameter and increasing dispensing pressure increase mechanical stresses and decrease cell viability after printing [110, 188]. Nanoparticles, either in the scaffold polymer or in the cells, may accentuate these
mechanical effects on cells. Printing resolution is also affected by nozzle diameter and printing pressure. Changes in biopolymer flow rate, printed line width, and pattern precision can alter important scaffold parameters, including porosity and elastic modulus [189-192].

The scaffold biopolymer properties impact the nano-bioprinting system by influencing nanoparticle manipulation after printing and printing resolution. Nanoparticle movement within a viscous material is a balance between the magnetic force that drives movement and the viscous drag that opposes movement [193]. Thus biopolymer viscosity, and any nanoparticle effects on biopolymer viscosity, may impact post-printing manipulation of cells and bioactive factors. The biopolymer viscosity, with and without nanoparticles, may also affect printing resolution. Previous studies with collagen, chitosan, and alginate scaffolds have shown that biopolymer viscosity is a function of shear rate, viscosity increases with biopolymer concentration, and cells may increase biopolymer viscosity [194]. Viscosity changes, including non-Newtonian effects, can change printed line width and dramatically affect printing of defined three-dimensional structures [189, 194, 195].

In this study, we initially hypothesized that combining of solid freeform fabrication system with magnetic nanoparticles would allow movement and imaging of cells and bioactive factors within three dimensional tissue scaffolds. Superparamagnetic iron oxide nanoparticles were bioprinted either in an alginate scaffold or inside endothelial cells using the multinozzle direct cell writing system. Cell viability was assessed for various nanoparticle and alginate concentrations at varied nozzle sizes and
printing pressures for cells printed either with nanoparticles in the alginate scaffold polymer or with nanoparticles in cells. The effect of cell dispensing parameters and scaffold biopolymer characteristics on cell viability, nanoparticle movement, and bioprinting patterning capabilities was evaluated. Nanoparticles in both alginate and endothelial cells were manipulated using a magnetic field. The influence of nanoparticles on alginate scaffold viscosity, as well as the effect of alginate viscosity on nanoparticle movement, was quantified. Lines and patterns were printed with and without nanoparticles in the alginate, and bioprinting resolution was measured. Finally, nanoparticles were patterned inside three-dimensional biopolymer scaffolds and imaged using a Micro Computed Tomography (MicroCT) scanner. Bioprinting of superparamagnetic iron oxide nanoparticles could help create more versatile tissue engineering structures, as well as improve our understanding of cell behavior in three-dimensional tissue culture. We now show that the hybrid nano-bioprinting system performs as well as the existing bioprinting system, but that nanoparticle manipulation is highly dependent on scaffold viscosity.

4.2 Materials and Methods

4.2.1 Chemical Formulation

Sodium alginate powder (FMCBioPolymer, Drammen, Norway) was dissolved in deionized water at 0.5, 1, 2 and 3% w/v concentrations. An ionic cross-linking solution was prepared by dissolving calcium chloride, CaCl₂ (BDH Chemicals, Poole, UK), in
deionized water. NanoArc magnetic iron oxide nanoparticles (Alfa Aesar, Ward Hill, MA) of 20-40 nm diameter were used in all experiments. Spherical nanoparticles of the given size were selected to obtain maximum uptake efficiency [196]. Sodium alginate-magnetic nanoparticle solutions were prepared by vigorously mixing sodium alginate with increasing concentrations of iron oxide nanoparticles to achieve a homogeneous nanoparticle distribution.

4.2.2 Cell Culture

Porcine aortic endothelial cells (PAEC) were isolated and cultured as previously described. Prior to printing, cells were gently mixed at a concentration of $1.5 \times 10^5$ cells/ml in sodium alginate solution to ensure uniform cell distribution. For magnetically labeled cells, PAEC in 100 mm tissue culture dishes were loaded with different nanoparticle concentrations and incubated at 37°C in a 5% CO$_2$ incubator for 24 hours. Cell nanoparticle uptake was confirmed by TEM. Our TEM images also suggest that the majority if not all cells take up nanoparticles.

4.2.3 Cell Dispensing System

A proprietary solid freeform fabrication–based direct cell writing system (Figure 24A) was developed to create three-dimensional tissue constructs by dispensing cells and biopolymers into predefined patterns [109, 110]. The direct cell writing system used in this study operates at room temperature and low-pressure conditions to facilitate deposition of living cells, growth factors, or other bioactive compounds in controlled
amounts with precise spatial positioning. Pneumatic microvalves (EFD, East Providence, RI) were used to apply printing pressures of 2, 5 and 40 psi [109, 110] (Figure 24B).

Sodium alginate was chosen as the scaffold biopolymer. Alginate-nanoparticle-cell mixtures with 0, 0.1, or 1.0 mg/ml nanoparticle concentration were printed with 410 and 250 µm nozzles. Control samples were dispensed in the system but without using nozzle tips. All samples were dispensed as 0.3 g of bulk material with a sample size of three, and each experiment was repeated a minimum of two times. Data presented are from one representative experiment. After dispensing, each sample was immediately submerged in a 5.0% w/v CaCl₂ cross-linking solution for 5 min, placed in supplemented medium, and returned to the incubator. Samples in the long-term study were cross-linked daily to maintain both cell immobilization and alginate structural integrity. Representative images of printed bulk samples and cell distribution in alginate bulk samples are presented in Figure 24C, D, E.
Figure 24. (A) Schematic of solid freeform fabrication-based cell writing system. (Reproduced from Fig. 1 of Chang et al (23).) (B) Pneumatic microvalve with nozzle tip printing pattern of nanoparticles mixed in alginate. (C) Printed bulk samples used for cell viability tests. (D) PAEC homogenously distributed in CaCl$_2$ crosslinked alginate. (E) Magnetically labeled PAEC homogenously distributed in alginate.

4.2.4 Cell Viability

Alamar blue quantitatively measures cell metabolic activity using an oxidation-reduction (REDOX) indicator that fluoresces and changes color in metabolically active cells [197]. Cross-linked alginate-cell solutions in 6 well plates were incubated with 2 ml supplemented medium and 200 μl Alamar blue solution (AbD Serotec Ltd, Oxford, UK). After 4 hours of incubation at 37°C in 5% CO$_2$ atmosphere, 100 μl of medium from each well was transferred into a 96 well flat-bottomed black assay plate, and fluorescence was measured at 535/590 nm in a GENios microplate reader. 3 x 10$^4$ cells were calibrated to a fluorescence intensity reading of 35000. Since the Alamar blue assay measures the mean
metabolic activity of the cell population, cell viability was confirmed using a Live/Dead assay (Invitrogen, Carlsbad, CA) as per manufacturer instructions.

4.2.5 Nanoparticle and Magnetically Labeled Cell Movement in the Scaffold

Bulk samples of 1.0 mg/ml magnetic nanoparticles in 0.5, 1.0 and 2% w/v alginate were printed using the direct cell writing system. A 1 inch diameter NdFeB magnet with a surface field of 6450 Gauss (K&J Magnetics, Jamison, PA) was placed under the 60 mm cell culture dishes. Specific patterns of nanoparticles and magnetically labeled cells were also printed using the cell dispensing system. A rectangular NdFeB magnet with a surface field of 6450 Gauss (K&J Magnetics, Jamison, PA) was used to move nanoparticles to a specified location either in a new pattern or while maintaining the original printed pattern. Movement of magnetic nanoparticles and the magnetically labeled cells by the applied magnetic field was imaged using a 4 Megapixel CCD camera (Alpha Innotech, San Leandro, CA).

4.2.6 Mathematical Evaluation of the Nanoparticle Movement

1.0 mg/ml magnetic nanoparticles in 1% or 2% w/v alginate were printed using the direct cell writing system at a fixed location (x = 2mm) from a 1 inch diameter NdFeB magnet (K&J Magnetics, Jamison, PA) (Figure 25A). Nanoparticle displacement along the magnet center line was imaged at 100 frames/second using a Nikon TS100 microscope. Nanoparticle velocity was calculated from the derivative of the transient displacement data.
Experimental nanoparticle velocity observations were compared to theoretical calculations. The net force induced on a superparamagnetic nanoparticle in a viscous medium by an externally applied magnetic field gradient is a balance of the magnetic force ($F_{mag}$) and the viscous drag ($F_{visc}$) [198, 199]:

\[ \vec{F}_{mag} = (\vec{m} \cdot \vec{\nabla}) \vec{B} \]  \hspace{1cm} (2)

\[ \vec{F}_{visc} = 3 \pi \eta d \vec{\nabla} \]  \hspace{1cm} (3)

where $\vec{m}$ is the total nanoparticle magnetic moment, which depends on the nanoparticle material and volume; $\vec{B}$ is the magnetic field; $\eta$ is the suspending fluid viscosity; $d$ is the nanoparticle diameter; and $\vec{\nabla}$ is the instantaneous nanoparticle velocity. For a one-
dimensional problem along the magnet centerline (x axis), the nanoparticle velocity $\vec{\nabla}$ at steady state can be obtained by balancing the forces from equations (2) and (3) as:

$$
\vec{\nabla} = \frac{M_s d^2}{\eta} \frac{dB}{dx}
$$

where $M_s$ is the particle saturation magnetization and $dB/dx$ is the magnetic field gradient along the center axis.

The field intensity was calculated along the center axis(x) of the cylindrical magnet using the following analytical expression [200]:

$$
B(x) = \frac{B_r}{z} \left[ \frac{x+1}{\sqrt{(x+l)^2+r^2}} - \frac{x}{\sqrt{x^2+r^2}} \right]
$$

where $B$ is the flux density at a point $x$ away from the pole face and parallel to the magnet axis, $l$ is the magnet length, and $r$ is the magnet radius. Note that the flux direction is normal to the pole surface along the axis. The residual induction of the permanent magnet is $B_r$ and is a characteristic of the magnet material. The magnetic flux density derivative, $dB/dx$ was calculated as:

$$
\frac{dB}{dx} = \frac{B_r}{z} \left[ \frac{(x+1)^2 + r^2}{{(x+l)^2+r^2}} - \frac{1}{2} \right] - \left( (x+1)^2 + r^2 \right)^{\frac{3}{2}} - \left( x^2 + r^2 \right)^{\frac{3}{2}} + x^2 (x^2 + r^2)^{\frac{3}{2}}
$$

Theoretical calculations were compared with experimental results by substituting appropriate materials properties for the magnet and nanoparticles used. The NdFeB had magnetic flux density $B_r = 14800$ G, radius $r = 25.4$ mm, and length $l = 12.7$ mm. Magnetic field along the center axis of the magnet reached a maximum of 5200 G near the pole and decreased to 4400 G 2mm away from the magnet (Figure 25B). Saturation magnetization $M_s$ of the iron oxide nanoparticles was taken from the literature, where it was measured to 66 emu/g using a SQUID magnetometer [201].
4.2.7 Viscosity Measurement

Viscosity of 1, 2 and 3% alginate was measured using a rotating viscometer (Brookfield Co. HBTD, Stoughton, MA) at 10, 20 and 50 rpm. 1 mg/ml and 5 mg/ml iron oxide nanoparticles were mixed with alginate and viscosity was measured.

4.2.8 Micro Computed Tomography Scan

A 1.5 mm X 1.5 mm area of 0.1 mg/ml magnetic nanoparticles was printed within a 5 mm X 5 mm X 2 mm 2% w/v alginate construct and imaged using a MicroCT scanner (SkyScan 1172). MicroCT allows non-destructive evaluation of the internal structure and composition of the sample based on changes in X-ray absorption. Image resolution was set at 2.16 µm with a filter of 1 mm aluminum. The rotation angle was 180° with a rotation step of 0.1°.

4.2.9 Statistical Analysis

Samples were statistically compared using Student’s t-test. Statistical significance was established at either p<0.05 (#) or p<0.01 (*). Two-way ANOVA was used to compare changes over time, with statistical significance established at p<0.0001.
4.3 Results

4.3.1 Viability of cells printed with magnetic nanoparticles in the alginate

Bioprinting magnetic nanoparticles along with cells in a biopolymer scaffold may provide an effective means to track and manipulate bioactive factors in tissue engineered structures. In this set of data printed caption in the figures represent cells printed with 250µm nozzle tip. We now show that while nanoparticles themselves slightly decreased endothelial cell viability, bioprinting had no significant effect (Figure 26A). At 0 and 12 hours after printing, cell viability did not change significantly for unprinted or printed cells with 0 or 0.1 mg/ml nanoparticles in a 1% w/v alginate solution. However, at 36 hours after printing, PAEC with 0.1 or 1.0 mg/ml nanoparticles were 16% or 35% less viable than cells without nanoparticles, respectively. The viability loss was independent of the printing process. Cell viability continued to decrease with time up to 60 hours after cell printing (ANOVA, p<0.0001). In a long term assay (Figure 26B), endothelial cell viability similarly decreased nearly 22% with 1.0 mg/ml iron oxide nanoparticles in the alginate 72 hours after printing compared to samples without nanoparticles (ANOVA, p<0.0001). No further cell viability decrease was observed from 72 hours to 144 hours, showing that cells maintained their viability following the initial nanoparticle toxicity effect.

Increased nanoparticle concentration decreased cell viability, but no additional decrease was observed with printing (Figure 26A). PAEC encapsulated in alginate with 1.0 mg/ml nanoparticles showed 20% lower viability than cells with 0.1 mg/ml nanoparticles and 36% lower than the control, suggesting a nanoparticle concentration
dependent effect on cell viability. This decreased viability was observed 36 and 60 hours after printing, but the printing process itself did not affect cell viability. To confirm that the Alamar blue measured cell viability, not a change in cell metabolism, a Live/Dead assay was performed on printed samples. The Live/Dead data agreed well with the Alamar blue results.
Figure 26. Endothelial cell viability decreased in a dose-dependent manner with magnetic nanoparticles in the alginate, but printing had no effect. (A) Cell viability for cells printed with 0, 0.1, and 1.0 mg/ml nanoparticles in 1% w/v alginate solution, assessed by Alamar blue fluorescence over time. (B) Long term cell viability for cells printed with 0 and 1.0 mg/ml nanoparticles in 1% w/v alginate solution, assessed by Alamar blue fluorescence up to 6 days after printing. (n = 3, # p < 0.05, * p<0.01 relative to no nanoparticle sample). Cells were printed with 250µm nozzle tip.
4.3.2 Effect of Alginate Concentration on Printed Cell Viability

We next investigated whether alginate concentration, which effectively alters biopolymer viscosity, affected printed cell viability. In this set of data printed caption in the figures represent cells printed with 250µm nozzle tip. Immediately following printing, there was a 20% viability decrease for cells printed with nanoparticles in 2% w/v alginate as compared to the 1% w/v alginate (Figure 27). 12 hours after printing, lower viability was also observed for control cells with nanoparticles in the 2% w/v alginate. This decreased cell viability for cells with nanoparticles in the 2% w/v alginate solution was no longer observed at later time points, primarily because cell viability decreased in the samples with nanoparticles in 0.5% or 1% alginate. Interestingly, in cell samples without nanoparticles, cell viability decreased for both control and printed cells without nanoparticles in the 2% w/v alginate solution at 36 and 60 hours (Figure 27C, D). Overall, cells without nanoparticles in the 0.5% and 1% w/v alginate solutions demonstrated an increase in Alamar blue fluorescence over time, which could represent increased cell number or increased cell metabolism. No cell samples in alginate with nanoparticles, and no cell samples in 2% alginate, showed this increase in viability with time. This effect also was independent of printing.
Figure 27. A higher viscosity alginate scaffold decreases cell viability, however effect timing depends on printing and nanoparticles. Cell viability for PAEC in 0.5, 1 and 2% w/v alginate and 0 or 0.1 mg/ml nanoparticles at 0 (A), 12 (B), 36 (C) and 60 (D) hours after printing. (n = 3, *p < 0.05, relative to 1% w/v alginate sample). Cells were printed with 250µm nozzle tip.
4.3.3 Effect of Cellular Nanoparticle Uptake on Printed Cell Viability

Magnetically labeled cells, internally loaded with iron oxide nanoparticles, could be used to track and move cells printed within a tissue engineered structure. The viability of nanoparticle loaded cells was examined after printing in 1% alginate and an initial dispensing pressure of 5 psi. Both control and printed samples without nanoparticles showed increased viability at timepoints up to 60 hours. However, a steep decrease in cell viability was observed from 0 to 36 hours for both control and printed cells loaded with either 0.1 or 1.0 mg/ml nanoparticles (Figure 28A). Printed cells showed the most dramatic change, with a 40% decrease in the Alamar blue fluorescence when compared to printed cells without nanoparticles at 36 hours. This viability change was in direct contrast to the lack of printing effect for samples with nanoparticles in the alginate. While early cell viability was significantly decreased, there was no significant change at timepoints after 36 hours, suggesting stabilization of the remaining cell population. When printing pressure was decreased to 2 psi, cell viability increased almost 20% (Figure 28B).
Figure 28. Cell viability decreased for cells loaded with 0.1 and 1.0 mg/ml nanoparticles and the decrease was accentuated by printing. PAEC viability for cells printed in 1% alginate at a dispensing pressure of (A) 5 psi and (B) 2 psi. (n = 3, # p < 0.05, * p<0.01 relative to no nanoparticle sample). Cells were printed with 250µm nozzle tip.

4.3.4 Effect of Nozzle Size and Printing Pressure on Cell Viability

Biofabrication conditions might significantly affect the nano-bioprinting system efficacy. We now show that while nanoparticles in the alginate slightly decreased endothelial cell viability, nozzle size had no significant effect (Figure 29A). At 0 and 12 hours after printing, cell viability did not change significantly for printed cells with 0, 0.1 and 1.0 mg/ml nanoparticles in a 1% alginate solution. However, 36 hours after printing, PAEC with 0.1 or 1.0 mg/ml nanoparticles were 16% or 35% less viable than cells printed without nanoparticles, respectively. The viability loss was independent of nozzle size. Cell viability continued to decrease up to 60 hours after cell printing (ANOVA, p<0.0001), however long term experiments showed no further cell viability decrease after 60 hours (data not shown).
Changes in the nozzle tip size might affect magnetically labeled cell viability during printing process. So nanoparticle loaded cell viability of cells printed with 250 and 410 µm nozzle tips was examined after printing. While viability was unchanged for printed cells without nanoparticles, viability decreased from 0 to 36 hours for printed cells loaded with either 0.1 or 1.0 mg/ml nanoparticles (Figure 29B). However, nozzle size did not affect cell viability. Nanoparticle loaded cells printed with either a 250 and 410 µm diameter nozzle demonstrated 36% viability loss compared to cells printed without nanoparticles at 36 hours. While early cell viability was decreased, there was no significant change after 36 hours, suggesting stabilization of the remaining cell population.

Increasing printing pressure from 5 psi to 40 psi decreased cell viability by 25% when nanoparticles were in the alginate (Figure 29C), and 26% for magnetically labeled cells (Figure 29D) immediately following bioprinting. Cell viability continued to decrease in a similar manner for both nanoparticle conditions and printing pressures. The combined effect of printing pressure and nanoparticles affected cell viability in an additive manner and at different times, suggesting no interaction between the two printing parameters.
Figure 29. Bioprinted endothelial cell viability decreased with nanoparticles and printing pressure, but nozzle diameter has no effect. Cell viability after bioprinting with 250 µm and 410 µm diameter nozzles, 1% alginate with 0, 0.1, and 1.0 mg/ml magnetic nanoparticles either (A) in the alginate or (B) loaded inside cells. Cell viability after bioprinting with 250µm diameter nozzle, 1% alginate at 5 and 40 psi printing pressure with 1.0 mg/ml nanoparticles either (C) in the alginate or (D) inside cells. (n = 3, # p < 0.05, * p<0.01 relative to 0 mg/ml nanoparticle sample).

4.3.5 Nanoparticle and Cell Manipulation inside the Alginate

Nanoparticles were magnetically manipulated within the alginate to determine if nanoparticles could be used to move bioactive factors after printing. 1.0 mg/ml nanoparticles were homogenously distributed in 1%, 2%, and 3% w/v alginate, printed in bulk samples, and left as a viscous liquid or crosslinked with calcium chloride to form a gel (Figure 30A, C, E; Figure 30G, I, K). Nanoparticles printed in either 1% or 2% w/v
alginate without calcium chloride moved towards the NdFeB magnet placed under the cell culture dish within a minute (Figure 30B, D; arrows indicate nanoparticles at the magnet edge). However, no nanoparticle movement was observed in the 3% w/v alginate solution, likely due to the high alginate solution viscosity (Figure 30F). When the samples were crosslinked with calcium chloride, nanoparticles similarly moved toward the magnet edge in the 1% and 2% w/v alginate, but not 3% alginate. (Figure 30H, J, L). However, the nanoparticles moved more slowly, and less spatial repositioning of nanoparticles was observed.

We next investigated if cells loaded with magnetic nanoparticles could be moved within the alginate biopolymer. PAEC magnetically labeled with nanoparticles were initially homogenously distributed in 0.5% and 1% w/v alginate (Figure 31A, E, I; higher magnification in Figure 31B, F, J). Magnetically labeled cells moved toward the NdFeB magnet placed under the cell culture dish (Figure 31C, G, K). At higher magnification, individual cells were seen at the magnet edge (arrows in Figure 31D, H and L). Isolated nanoparticles can also be seen in the alginate, which are likely artifacts of incomplete nanoparticle removal from the cell solution when it was mixed with alginate. Magnetically labeled cells continued to cluster at the magnet edge in the cross-linked alginate, but no movement was observed in alginate concentrations higher than 1%.
Figure 30. Bioprinted nanoparticles in the alginate scaffold move towards the magnet in a manner dependent on scaffold viscosity. 1.0 mg/ml nanoparticles homogeneously distributed in 1%, 2% and 3% w/v alginate (A, C, E) moved towards the NdFeB magnet for the 1% and 2%, but not 3%, alginate (B, D, F). When samples were crosslinked in CaCl$_2$ (G, I, K), nanoparticles moved more slowly and less nanoparticle movement was observed (H, J, L). Dash lines indicate magnet location. Scale bar is 1 mm.
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Figure 31. Magnetically labeled cells can be moved within the alginate scaffold using a magnet. PAEC loaded with 1 mg/ml nanoparticles homogenously distributed in 0.5 and 1% w/v alginate (A, E; higher magnification B, F) and in 0.5% alginate crosslinked with CaCl₂ (I higher magnification J). Cells moved toward an NdFeB magnet placed under the culture dish (C, G, I; higher magnification D, H, L). Dashed lines indicate magnet location. Arrows indicate magnetically labeled cells accumulated at the magnet edge.

Specified patterns of nanoparticles and magnetically labeled cells were printed and moved using a magnetic field. 1% alginate with iron oxide nanoparticles was printed in a pattern (Figure 32A), and a magnetic field was used to move the nanoparticles to the printed pattern tips (Figure 32B). Basic shapes (lines and rectangles) of either nanoparticles (Figure 32C, D; Figure 32G, H) or magnetically labeled cells (Figure 32E, F) were moved to new locations while maintaining the original pattern.
Figure 32. Printed shapes of nanoparticles or magnetically labeled cells in alginate were moved to new locations using a magnetic field. (A, B) 1% alginate with nanoparticles was printed in a specified pattern. Nanoparticles were moved to the pattern tips using a magnetic field. (C, D) Nanoparticles or (E, F) magnetically labeled cells were printed in a 600 µm thick line in a 25x25 mm 2% alginate square. The printed line pattern was moved using a magnetic field. Arrow shows the magnet location. (G, H) Nanoparticles printed in a rectangle were moved towards the magnet while maintaining the rectangular pattern.

4.3.6 Effect of Nanoparticles on Alginate Viscosity

Biopolymer scaffold viscosity affects printing resolution, therefore alginate viscosity at different concentrations and with nanoparticles was measured. Alginate viscosity increased with alginate percentage and decreased with rotational velocity. At 20 rpm, viscosity increased from 400 cP for 1% alginate to 1250 cP for 2% alginate and 8000 cP for 3% alginate (Figure 33A-C). Alginate viscosity decreased more than 25% with increasing velocity (strain rate) for all concentrations, with 3% alginate showing the
most dramatic non-Newtonian properties. 1.0 mg/ml and 5.0 mg/ml iron oxide nanoparticles did not significantly affect 1% alginate viscosity, at least within the measurement capability of the system (Figure 33A). However, in 2% (Figure 33B) and 3% (Figure 33C) alginate, 5.0 mg/ml nanoparticles resulted in a statistically significant increase in alginate viscosity (p<0.05).

Figure 33. Alginate viscosity decreased with velocity, but only high nanoparticle concentrations increased viscosity. Viscosity with 0, 1.0, and 5.0 mg/ml nanoparticles added for (A) 1% alginate, (B) 2% alginate, and (C) 3% alginate. (n=3, # p< 0.05).

4.3.7 Effect of Alginate Viscosity and Nanoparticle Cluster Size on Nanoparticle Velocity

Nanoparticle velocity in the alginate biopolymer was quantified as a function of alginate viscosity and nanoparticle cluster size. Nanoparticle velocity was four times faster in 1% alginate than 2% alginate (Figure 34A). Due to limited testing length, the
nanoparticles did not reach a constant velocity. Instead, they accelerated at $0.385 \text{ mm/s}^2$ in 1% alginate, and much slower at $0.088 \text{ mm/s}^2$ in 2% alginate. While ideally nanoparticles would be monodispersed in the alginate biopolymer, particles aggregated in clusters, particularly when a magnetic field was applied. Velocities for three nanoparticle cluster sizes were compared in 2% alginate. The larger cluster sizes moved faster in the alginate, which agreed with the theoretical calculation that nanoparticle velocity increases with the square of particle diameter. In our experiments, 200 µm sized clusters moved five times faster than 50 µm sized cluster when the nanoparticles were 0.9 mm from the magnet (Figure 34B). Experimentally determined nanoparticle cluster velocities showed good agreement with calculated velocities (Table 3).

Figure 34. Nanoparticle velocity increased at low alginate concentrations and for larger nanoparticle clusters (A) Nanoparticle velocity in 1% and 2% alginate biopolymer, and (B) Nanoparticle velocity as a function of agglomerated nanoparticle cluster size.
Table 3. Comparison of measured nanoparticle velocity with calculated velocity.

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4.3.8 Effect of Nanoparticles on Printing Resolution

Nanoparticles may alter biopolymer flow rate, and therefore affect bioprinting resolution. Lines were printed with and without nanoparticles with 250 µm and 410 µm nozzles at 2, 3.5, and 5 psi printing pressure (Figure 35A). Printed lines were the same width as the nozzle diameter at the low 2 psi pressure. As printing pressure increased to 3.5 and 5 psi, printed line width increased linearly to more than twice the nozzle diameter (Figure 35B). However, the presence of nanoparticles in alginate did not change printed line width (Figure 35C). Printing resolution was maintained with nanoparticles, as shown by the complex patterns printed with (Figure 36B, D, F) and without (Figure 36A, C, E) nanoparticles.
Figure 35. Printing pressure increased line width, but nanoparticles did not affect printing resolution. (A) Lines printed with and without nanoparticles using 250 and 410 µm nozzles and 2, 3.5, and 5 psi printing pressures. (B) Measured line width as a function of nozzle size and printing pressure. (C) Measured line width as a function of printing pressure, with and without nanoparticles, for the 410 µm nozzle.
4.3.9 MicroCT Scan of 3D Deposited Tissue Scaffold

Magnetic nanoparticles printed within three-dimensional alginate scaffolds were imaged by MicroCT to determine if nanoparticle printing would allow non-invasive tracking of bioactive factors and cell location in tissue engineering structures. A nanoparticle-alginate prepolymer solution was encapsulated in alginate biopolymer solution using layer-by-layer deposition with the solid freeform fabrication based direct cell writing system. Printed nanoparticle clusters are clearly visible by MicroCT scan of the three-dimensional tissue scaffold (arrows, Figure 37).

Figure 36. Printing resolution for complex patterns was maintained with nanoparticles in the alginate. Shapes printed with alginate (A, C, E) and alginate with nanoparticles (B, D, F) were imaged using a CCD camera.
Figure 37. Nanoparticles printed within a three-dimensional alginate biopolymer are visible by MicroCT. Images represent sample cross sections in the (A) translational plane, (B) coronal plane and (C) sagittal plane. The red and blue lines on the translational plane (A) show the sagittal and coronal plane cuts, respectively (B, C). The green line in the sagittal and coronal plane views represents the translational plane cut. Arrows indicate nanoparticles. Scale bar is 500 µm.

4.4 Discussion

Enhanced non-destructive imaging of cellular and biochemical interactions within three-dimensional tissues would advance knowledge of tissue development, and the ability to precisely pattern cells and bioactive factors throughout the tissue growth process would improve fabrication of complex tissues. A combination of bioprinting, which allows initial patterning, and superparamagnetic nanoparticles, which allow tracking and re-patterning, could help realize these tissue engineering goals. Furthermore the effect of biofabrication parameters on bioprinting efficacy is critical for implementation of novel cell deposition systems in tissue engineering. We now show that
magnetic nanoparticles can be bioprinted for tissue engineering applications. Nanoparticles in the biopolymer scaffold or inside cells decrease printed cell viability, but that nanoparticles do not accentuate the effects of nozzle size and printing pressure. In addition, while printing cells loaded with nanoparticles did decrease cell viability, the viable cells were stabilized shortly after printing. Magnetically labeled biofactors and cells could be moved within the alginate structures in the presence of an externally applied magnetic field, or imaged non-destructively using MicroCT. After printing, biopolymer viscosity and nanoparticle cluster size control nanoparticle velocity within the scaffold in response to a magnetic field. Since lower nanoparticle concentrations do not significantly affect biopolymer viscosity, printing resolution and patterning capabilities are maintained for the nano-bioprinting system. These results support appropriate biofabrication parameter selection for the hybrid nano-bioprinting system, which enables initial patterning followed by non-destructive imaging and re-patterning of cells and bioactive factors in tissue engineered constructs.

Sodium alginate was used in all experiments because it is non-toxic, remains a viscous liquid at room temperature, and cross-links to form a gel under mild conditions. However, endothelial cells are unable to specifically interact with alginate, which prevents cell anchorage and attachment in the biopolymer [202]. The cells have a rounded morphology because they are encapsulated in, not adhered to, the polymer scaffold. Since the printing system operates at room temperature, we are currently unable to print polymers such as collagen, to which the cells would attach, because collagen solidifies into a stiff gel at room temperature. In our experiments, alginate is
advantageous because it maintains cell number without proliferation, which allows improved observation of cell death. As a future work of this study, we suggest to incorporate a cooling system into our bioprinting device to print collagen gels. It will then make it possible to study cell viability and proliferation after cells printed with nanoparticles have attached to the scaffold.

By bioprinting nanoparticles in the biopolymer, bioactive factors such as growth factors, antibodies, drugs, and genes conjugated to the magnetic nanoparticles can be precisely patterned within a three-dimensional scaffold. Chemical coupling via amide or ester bonds has been used by others to conjugate bioactive factors to iron oxide nanoparticles. Linker molecules, including 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), or N-hydroxysuccinimide were used to attach targeting ligands and proteins to nanoparticle surfaces [4]. Insulin, lactoferrin and ceruloplasmin were successfully conjugated to superparamagnetic iron oxide nanoparticles using EDCI. These nanoparticles were then targeted to cell surface receptors, thereby avoiding endocytosis, to achieve tissue and cell-specific drug targeting [67, 68]. Magnetic nanoparticles have also been dually conjugated, both with a ligand specific for a target cancer cell receptor and a cancer therapy drug. For instance, a radiolabeled anti-vascular endothelial growth factor (VEGF) monoclonal antibody was conjugated to magnetic nanoparticles to both target and deliver radiation therapy to liver cancer [203]. By chemically conjugating bioactive factors to the nanoparticles, it would be possible to initially pattern the bioactive factors in the scaffold
and then move them during tissue growth. This technique could, for example, provide endothelial cells with a changing growth factor gradient to promote angiogenesis.

In the current study, we observed decreased viability for cells exposed to both low and high nanoparticle concentrations in the alginate, independent of bioprinting. However, in our previous studies, endothelial cells viability was largely preserved in two-dimensional culture up to an iron oxide nanoparticle concentration of 0.5 mg/ml [181]. This apparent contradiction might be related to differences in two- and three-dimensional endothelial cell culture. In two-dimensional cultures, endothelial cells form a stable, confluent monolayer, whereas these same cells form tubes when grown in a three-dimensional matrix [204]. The three-dimensional angiogenic endothelial cell phenotype may be more susceptible to nanoparticle toxicity than the two-dimensional monolayer. Cells may be able to uptake more nanoparticles in the three-dimensional alginate structure since they are exposed to nanoparticles on all sides. In two-dimensional culture, cells interact with nanoparticles only at their apical surface. The effect could also be specific to the alginate scaffold since cells do not attach to the alginate. These attachment-dependent cells may experience changes in critical cell functions when encapsulated, which coupled with functional changes caused by nanoparticle uptake, may enhance nanoparticle cell toxicity [205]. Interestingly, long term tests showed that cell viability is stable after 72 hours, perhaps because all available nanoparticles in the vicinity of a cell have already been taken up.

The printing process itself did not impact endothelial cell viability when nanoparticles were mixed with the alginate. We hypothesize that cell viability is
preserved because nanoparticles are free to move within the alginate without damaging cells during printing. In direct contrast, when nanoparticles were loaded inside cells, there were fewer viable printed cells than control cells but this decrease in viability was found to be related to the printing pressure. The system operates at printing pressures ranging from 1 - 40 psi. The majority of our experiments were conducted at a printing pressure of 5 psi, which was shown in previous studies to maintain cell viability. Only at pressures greater than 20 psi did cell viability decrease [110]. As dispensing pressure was lowered to 2 psi, the forces imposed on the cell as it moved through the nozzle decreased, which likely increased cell viability. However the printing process at 2 psi dispensing pressure took more than twice as long, so for automated mass production, higher printing pressures such as 5 psi might still be preferred with the known loss in cell viability. At both pressures, cells without magnetic nanoparticles recovered in time from the mechanical perturbation and eventually reached a steady state condition.

When cells uptake nanoparticles, they form vacuoles which disrupt the cell cytoskeleton [68]. Cells with a disrupted cytoskeleton may experience increased damage due to printing-induced forces. Alternatively, the nanoparticles inside cells may be more likely to damage the cell membrane through direct shear effects during the printing process. As the cells are pushed through the nozzle, the nanoparticles may break through the cell membrane or the nucleus, causing irreversible damage. It is also possible that cells loaded with nanoparticles are already in a state of internal stress, perhaps due to reactive oxygen species (ROS). Intracellular ROS generation is hypothesized to increase with nanoparticle uptake, leading to protein, DNA and tissue injury and eventual cell
death [113]. The addition of mechanical stress from the bioprinting process may be more toxic when cells are already biochemically stressed by the internalized nanoparticles prior to the printing process. Both printing parameters and nanoparticle conditions will need to be optimized to minimize cell death.

Nozzle size did not affect cell viability, whether cells were printed with nanoparticles in the alginate or inside the cells. These results agree with previous research suggesting that only the smallest nozzle size (150 µm) decreased cell viability after printing. We had previously hypothesized that nanoparticles might effectively decrease nozzle size during printing, since the nanoparticles are more rigid than alginate or cells. However, cell viability appears to be preserved because nanoparticles are free to move within the alginate or cells without damaging the cell membrane during printing. We had also postulated that nanoparticles in cells, which increase intracellular reactive oxygen species and put cells in a stressed state [68], might sensitize cells to increased shear stress experienced with smaller nozzle sizes. Since no effect was observed, nozzle size does not appear to be significant at larger nozzle sizes. However, there might be a nanoparticle-induced cell viability loss at smaller nozzle sizes.

In contrast to nozzle size, higher dispensing pressures decreased cell viability for cells printed with and without nanoparticles. Cell viability decreased immediately after printing due to the higher pressure of 40 psi and then continued to decline due to nanoparticle effects. The early viability loss was effectively the same for cells printed with and without nanoparticles, suggesting that the printing pressure effect was not accentuated by nanoparticles. These results are comparable to previous studies, in which
printing pressure similarly affected a hepatocyte cell line (HepG2) viability [110]. The hepatocytes recovered to the original viability level by 24 hours after printing, suggesting that cell metabolic activity was only temporarily compromised by higher printing pressures. We did not observe this recovery effect, perhaps because primary endothelial cells were used rather than a hepatic carcinoma cell line.

Cell toxicity could alternatively be decreased, while still allowing cell tracking, by attaching bioconjugated nanoparticles to the cell membrane. However it might be difficult to manipulate cells with externally-attached nanoparticles. The nanoparticles may detach during printing due to mechanical forces as the cell moves through the nozzle, or the nanoparticles may detach during cell movement through the scaffold due to viscous drag. Furthermore, attaching nanoparticles to cells via a surface receptor could activate unwanted intracellular signaling, or prevent the cell from using that receptor to perform a particular function. While keeping these challenges in mind, as a future work, we suggest investigating whether printing and moving cells with membrane-attached nanoparticles is feasible and maintains cell viability.

Our studies revealed that cell viability decreased for samples printed in 2% w/v alginate as compared to 1.0% w/v alginate with and without any nanoparticles. The high solution viscosity may have exposed cells to higher printing forces during the cell dispensing process. Cell membranes are highly fragile to mechanical loads, and excessive membrane perturbation can lead to cell death [206]. The initial cell viability change observed from 0 to 12 hours in samples with nanoparticles in 2% alginate suggests that nanoparticles further increased the biopolymer viscosity. At later times (36 and 60 hours
after printing), the presence of nanoparticles inside the alginate overcame this initial 2% alginate cell viability decrease, and there was no difference among the different alginate samples. Even the cells without nanoparticles demonstrated decreased viability in the long term due to the 2% alginate, which was in contrast to the recovery process for cells in 1% alginate. This suggests that endothelial cell health is compromised in the stiffer gel, however additional studies are needed for confirmation.

While cells in our experiments showed lower viability in stiffer gels, other studies have shown that cells prefer stiffer substrates. In two-dimensional culture, cells form large, stable focal adhesions on stiff substrates, whereas cells form irregularly shaped, dynamic adhesions on softer substrates [207]. However for three-dimensional cell studies, cell migration speed and viability may depend not only on the substrate stiffness but also substrate adhesivity or cell–matrix adhesion availability [208]. Peyton and Putnam [209] found that when cell adhesiveness was reduced using an integrin-blocking antibody, the maximum cell migration speed shifted from stiffer to softer Matrigel substrates. Our printed cells are in three-dimensional alginate gels to which they do not attach, therefore it is possible that unattached cells are more viable in softer alginate gels. There are rich opportunities for future studies to address mechanisms underlying these distinct differences in cell viability and migration in two- and three-dimensional environments and the relationship between substrate stiffness and cell-matrix adhesion.

The hybrid nano-bioprinting system allows initial nanoparticle and cell patterning by computer-controlled printing, after which nanoparticles and cells can be moved to a new location either in the initial pattern or in a new pattern defined by the magnetic field.
We now also show that nanoparticle movement depends on alginate viscosity. Nanoparticles moved towards the NdFeB magnet in 1% and 2%, but not 3% alginate. As seen from equation (4), the nanoparticle velocity inside the alginate is inversely proportional to the medium viscosity and directly proportional to the magnetic field gradient. So in a higher viscosity biopolymer, a stronger magnetic field will be needed to move the same nanoparticle. Even though nanoparticles did not noticeably move in 3% w/v alginate, and nanoparticle movement decreased with crosslinking, it may be possible to move these nanoparticles in the more viscous biopolymer with a stronger magnet. These nanoparticles could even be removed prior to tissue implantation, which would decrease any potential negative effects in vivo. When a magnet was placed next to a nanoparticle-alginate scaffold, nanoparticles moved completely out of the alginate and attached to the magnet.

Alginate exhibits non-Newtonian shear-thinning behavior, meaning that solution viscosity decreases with increasing shear rate [210]. Alginate viscosity depends on alginate concentration and the number of monomers in alginate chains (molecule length) [211]. In general, liquid viscosity increases upon the addition of rigid spherical or non-spherical particles because particles disturb the fluid flow. With greater particle clustering, or more open cluster structure, a larger change in liquid viscosity is observed [212]. In our experiments, nanoparticles only increased alginate viscosity for high alginate concentrations and very high nanoparticle concentrations. At these high concentrations, nanoparticles likely form large clusters and impact liquid rheology. This viscosity increase could amplify the mechanical forces experienced by cells during
printing and thus impact cell viability, but it would enhance printing resolution [194]. Since our printed nanoparticle concentrations were 1.0 mg/ml and lower, alginate viscosity is not significantly affected within our system.

Experimental magnetic nanoparticle movement depended on particle size and shape, suspending fluid characteristics, and the external magnetic field strength, which agreed well with theoretical calculations [199]. Discrepancies between experiment and theory could be related to the testing field length, nanoparticle clustering, and local nanoparticle effects on fluid viscosity. Due to limitations in the experimental setup size, nanoparticles did not reach a terminal velocity but continued to accelerate as they approached the magnet. Since the velocity was calculated for nanoparticles with no net applied force, and therefore no acceleration, the experimental conditions did not exactly match the theoretical case. The mathematical model also assumed nanoparticles were single spheres. However, under the effect of magnetic field, nanoparticles aligned, rotated and formed linear chain-like aggregates. The diverse nanoparticle shapes and sizes affected similarity between experiment and theory. Finally, high nanoparticle concentrations and clustering in response to the magnetic field may have changed the local fluid viscosity [213]. Large nanoparticle clusters might even have prevented the movement of smaller nanoparticles by disturbing the flow stream, further deviating experimental from calculated velocities. While inconsistencies existed between the experimental conditions and the model assumptions, the measured and calculated velocities were adequately close to justify using this model to determine nanoparticle movement in a tissue engineering scaffold in response to a magnetic field.
The inclusion of nanoparticles in the alginate biopolymer did not reduce printing resolution. For alginate with and without nanoparticles, line width increased with nozzle diameter and printing pressure in accordance with the literature [189-191, 194]. Existing biofabrication procedures could be used to regulate the size, shape, porosity, and mechanical properties of a tissue engineering construct created with the nano-bioprinting system. The slight increase in biopolymer viscosity with nanoparticles would enhance printing resolution, but might also slightly increase the scaffold elastic modulus. This effect would require further investigation, if high nanoparticle concentrations are used.

Magnetically labeling cells allows non-destructive imaging by MicroCT in the 3D tissue engineering scaffold. The intracellular nanoparticle loading should be optimized to obtain maximum signal intensity while at the same time protecting cell viability. Our studies showed that it may be possible to qualitatively determine the nanoparticle or magnetically labeled cell density at a given location by MicroCT signal intensity. A 6 fold increase in signal intensity was observed for 100,000 cells loaded with 1.0 mg/ml iron oxide nanoparticles as compared to the same cell number loaded with 0.1 mg/ml nanoparticles. However, excess nanoparticles that were not taken up by cells could also increase the MicroCT signal intensity. Nanoparticle loading parameters should be selected to minimize free nanoparticles, and any excess nanoparticles should be thoroughly washed away prior to cell printing. Magnetically labeled dead cells will light up on the MicroCT scans, which may decrease live cell tracking efficacy. An additional assay, such as Alamar blue or Live/Dead, may be needed to differentiate live from dead cells. Live magnetically labeled cells would also respond to biochemical signals by
proliferating, moving, and forming three-dimensional structures, which should help distinguish them from dead cells in MicroCT images.

While cell viability was decreased with nanoparticle loading and printing, there is still potential by optimizing nanoparticle concentration and printing parameters to bioprint magnetically labeled cells. In tissue engineering, the development of complex three-dimensional tissues requires various cell types, such as smooth muscle and endothelial cells for vascular systems or hepatocytes and sinusoidal endothelial cells for liver. However cell-cell interactions are difficult to manipulate in coculture systems, even in two-dimensional cultures. Ito et al. [111] used magnetic force to place magnetically labeled cells onto target cells and promote heterotypic cell-cell adhesion. The solid freeform cell writing system could enable assembly of three-dimensional patterned tissue engineering constructs with various magnetically labeled cell types. Through MicroCT, both bioactive factors and cells could be non-invasively imaged within the tissue engineering scaffold, which would allow longitudinal studies of tissue development. This hybrid nano-bioprinting technique, which uses a combination of bioprinting and active magnetic patterning, could dramatically impact our ability to understand and re-create complex tissue development.

All of our experiments used a relatively low cell concentration in the alginate polymer. Higher cell density may be required to achieve critical cell mass for tissue engineering applications. Based on the studied parameters, including nanoparticle concentration, alginate viscosity, and nanoparticle movement within the alginate, we do not anticipate significant changes in printed cell viability or bioprinting quality with
higher printed cell concentrations. Neither increasing nanoparticle concentration nor decreasing nozzle diameter diminished cell viability. This suggests that more cells could be incorporated in the alginate without causing cell damage through cell-cell or cell-nozzle interaction during printing. A higher cell concentration may increase alginate viscosity, which in turn may impact cell viability. However, increasing nanoparticle concentration in the alginate had only a minor effect on alginate viscosity; therefore we do not anticipate a large change in alginate viscosity with increased cell density. Higher cell concentration may also decrease cell movement in the alginate in response to a magnetic field, since larger nanoparticle clusters moved more slowly. However, with time and large magnetic forces, cell movement should still be possible. Finally, since bioprinting resolution was unchanged with nanoparticles in the alginate, we similarly expect that resolution will be maintained with higher cell concentrations.

We have shown that a solid freeform fabrication system can be used to create magnetically functionalized three-dimensional tissue scaffolds, which can be manipulated after printing using a magnetic field. This new hybrid nano-bioprinting system enables enhanced tissue engineering capabilities, including imaging and repositioning cells and bioactive factors after initial deposition. Our data indicate that nanoparticle inclusion within a solid freeform fabrication system is unlikely to significantly change biofabrication parameters unless extremely high nanoparticle concentrations are used. A theoretical model for superparamagnetic nanoparticle movement in response to a magnetic field can be used to approximate nanoparticle repositioning within the scaffold. As a future work, we suggest to improve printed magnetically labeled cell viability by
exploring different nanoparticle sizes and shapes, as well as attaching nanoparticles to the cell membrane. Nanoparticles might be conjugated with bioactive factors, and cells might be printed in collagen gels with a cooled printing system. Finally, complex magnet arrangements might be used to control cell movement and patterning after printing in the tissue engineered construct.
CHAPTER V: CONCLUSIONS AND FUTURE WORK

5.1 Summary of Principle Findings

Superparamagnetic iron oxide nanoparticles are used in diverse applications, including optical magnetic recording, catalysts, gas sensors, targeted drug delivery, magnetic resonance imaging and hyperthermic malignant cell therapy. Nanoparticle combustion synthesis has significant advantages, including improved nanoparticle property control and commercial production rate capability with minimal post-processing. In the current work, superparamagnetic iron oxide nanoparticles were produced by flame synthesis using a coflow flame. Final iron oxide nanoparticle morphology, elemental composition, and particle size was controlled by changing flame configuration (diffusion and inverse diffusion), flame temperature, and additive loading.

The synthesized nanoparticles were primarily composed of two well known forms of iron oxide, namely hematite $\alpha$Fe$_2$O$_3$ and magnetite Fe$_3$O$_4$. We found that the synthesized nanoparticles were smaller for an inverse diffusion flame as compared to a diffusion flame configuration when CH$_4$, O$_2$, Ar and N$_2$ gas flow rates were kept constant. To investigate the effect of flame temperature, CH$_4$, O$_2$, Ar gas flow rates were kept constant, and N$_2$ gas was added as a coolant to the system. TEM analysis of iron oxide nanoparticles synthesized using an inverse diffusion flame configuration with N$_2$ cooling demonstrated that particles no larger than 50-60 nm in diameter can be grown, indicating that nanoparticles did not coalesce in the cooler flame. Raman spectroscopy
showed that these nanoparticles were primarily magnetite, as opposed to the primarily hematite nanoparticles produced in the hot flame configuration. To understand the effect of additive loading on iron oxide nanoparticle morphology, an Ar stream carrying titanium-tetra-isopropoxide (TTIP) was flowed through the outer annulus along with the CH$_4$ in the inverse diffusion flame configuration. When particles were synthesized in the presence of the TTIP additive, larger monodispersed individual particles were synthesized as observed by TEM. So with this study we show that iron oxide nanoparticles of varied morphology, composition, and size can be synthesized and controlled using flame synthesis method by varying flame configuration, flame temperature, and additive loading.

Then we compared flame synthesized iron oxide nanoparticle interaction with endothelial cells to commercially available iron oxide nanoparticles. We selected a heterogeneous mixture of 6-12 nm diameter hematite and magnetite nanoparticles with superparamagnetic properties, which were produced by an inverse diffusion flame configuration with N$_2$ cooling. Endothelial cell transmission electron microscopy and scanning electron microscopy, confirmed by energy dispersive spectroscopy, demonstrated that flame synthesized nanoparticles were ingested into cells in a similar manner to commercially available nanoparticles. The flame synthesized particles showed no statistically significant toxicity difference from commercially available nanoparticles, as measured by Live/Dead assay, Alamar blue, and lactase dehydrogenase release. Neither type of nanoparticle affected cell proliferation induced by fibroblast growth factor-2. These studies suggested that the iron oxide nanoparticles we synthesized by
flame synthesis are equally non-toxic to cells as the commercially available ones, and therefore are comparable to commercially available nanoparticles for biological applications.

We also showed that when iron oxide nanoparticles were uptaken by PAEC, they localized inside the cell cytoplasm as aggregated clusters. Nanoparticle uptake in the cell cytoplasm increased ROS formation in cells during the first three hours after uptake. ROS formation was found to induce actin cytoskeleton disruption, and actin fibers started to elongate after 4 hours. Actin fiber elongation continued for 12 hours after uptake. ROS blockers also blocked actin cytoskeleton elongation, which points to a coupled reaction between ROS formation and actin cytoskeleton disruption. We demonstrated for the first time that iron oxide nanoparticles result in endothelial cell ROS formation in a dose dependent manner, and this ROS formation leads to actin cytoskeleton disruption. We further showed that we can block the negative effects of the nanoparticles on the cell cytoskeleton by blocking the ROS formation.

After synthesizing iron oxide nanoparticles that were comparable to commercially available ones, and studying nanoparticle toxicity, we investigated possible applications of iron oxide nanoparticles in tissue engineering. We developed a new hybrid nano-bioprinting technique that facilitates manipulation and tracking of cells and bioactive factors within a three-dimensional tissue construct. This technique combined the initial patterning capabilities of syringe-based cell deposition with the active patterning capabilities of superparamagnetic nanoparticles. Superparamagnetic iron oxide nanoparticles, either in the alginate biopolymer or loaded inside endothelial cells, were
bioprinted using a solid freeform fabrication direct cell writing system. Bioprinting did not impact cell viability when nanoparticles were in the alginate, however it did have a pressure-dependent effect when nanoparticles were inside cells. Nanoparticles did not change bioprinting resolution. Nanoparticles in the alginate and loaded in cells were moved using an external magnet, and nanoparticle velocity depended on nanoparticle diameter and scaffold viscosity. These results agreed with a mathematical model of nanoparticle movement. Finally, nanoparticles in the alginate and in cells were imaged by MicroCT. The hybrid nano-bioprinting method enabled us to non-invasively manipulate and track nanoparticles and cells within tissue engineering structures.

5.2 Suggested Future Work

5.2.1 Nanoparticle Synthesis

With the recent awareness of nanoparticles and their promising properties, there is renewed interest in their inexpensive and flexible manufacture. Flame reactors are routinely used to manufacture more than 90% in volume and value of nanoscale commodities today [214]. As a result, they have high potential for making a spectrum of oxide nanoparticles. In this thesis we designed a laboratory scale coflow flame synthesis setup to synthesize iron oxide nanoparticles with controlled size and morphology. We now suggest as a future work to investigate progress scale-up. Specifically iron oxide nanoparticle synthesis in several diffusion flame reactors of increasing size should be systematically studied. These reactors should be operated at the same precursor and fuel
flow rates over a range of oxidizer flow rates. For each of the reactors covering the turbulent and laminar regimes, a single operation line relating the average product particle diameter to burner outlet conditions should be developed for the three reactors covering the laminar and turbulent flame regimes. Based on this, process scale-up and an operation diagram can be developed for the iron oxide nanoparticle synthesis in coflow diffusion flames.

5.2.2 Cellular Uptake and Toxicity Studies

In this thesis, we showed that flame synthesized nanoparticles are equally biocompatible as commercially available ones. However, biocompatible coatings may reduce nanoparticle toxic effects and further surface functionalization with bioactive factors would make them ideal candidates for several biomedical applications [4]. For this reason, as a future work we suggest studying possible methods to coat and functionalize nanoparticle surfaces.

We also showed that reactive oxygen species formation and actin cytoskeleton disruption are two mechanisms working in a coupled manner for nanoparticle toxicity. However further studies should be performed to investigate whether nanoparticle uptake changes cellular response to biological stimuli. Considering that endothelial cells line the inner blood vessel surface, one stimulus is blood flow and the resultant shear stress. As future work we suggest investigating changes in cell shear stress response when cells are loaded with iron oxide nanoparticles. Below you can find our detailed plan of action to address these two topics.
5.2.2.1 Functionalization of Nanoparticle Surfaces for Biological Applications

Suggested future work includes coating and functionalizing nanoparticle surfaces for biological applications, including specific cell targeting and bioactive factor delivery. As previously mentioned, iron oxide nanoparticles can be conjugated to bioactive factors by chemical coupling methods via amide or ester bonds [4]. We suggest using linker molecules, including 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide hydrochloride (EDCI), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), or N-hydroxysuccinimide to attach targeting ligands and proteins to nanoparticle surfaces. Then nanoparticle endothelial cell toxicity can be assessed using the toxicity assays used in this thesis and results can be compared to bare iron oxide nanoparticles. Also the nanoparticle effects on the cellular cytoskeleton, specifically actin fiber elongation and ROS formation should be investigated. It is expected that when nanoparticles are coated with biocompatible coatings it would be possible to increase the nanoparticle dose used in the system. It is also expected that these nanoparticles will lead to less defects in the cellular cytoskeleton and a decrease in ROS formation. Also by using these functionalized nanoparticles in the hybrid nano-biofabrication system, it would be possible to initially pattern the bioactive factors in the scaffold and then move them during tissue growth while at the same time tracking them. This technique could, for example, provide endothelial cells with a changing growth factor gradient to promote angiogenesis.
5.2.2.2 Magnetically Labeled Cell Response to Shear Stress

We further suggest studying the effect of nanoparticle-induced actin cytoskeleton disruption under mechanical stimuli. We performed preliminary studies investigating magnetically labeled cell behavior under applied shear stress conditions. Endothelial cell elongation and alignment in the fluid flow direction was first reported in 1972 by Flaherty et al. [215] and in vitro in the 1980’s by Dewey et al. [216]. The response was found to be related to shear stress changes, as the cells would accommodate the shear stress quickly and return to basal morphology in static conditions[217]. The fluid flow effect was further shown to depend on shear stress intensity [218, 219]. These studies were all under laminar shear stress in an in vitro setting, but endothelial cell elongation and alignment has also been observed in vivo [220, 221]. The changes are also seen in the internal cell structures, namely the cytoskeleton and the actin filaments. Early actin fiber studies showed that they also aligned in the fluid shear stress direction [222-225].

We used a rectangular parallel plate flow chamber (GlycoTech Corporation) designed for use with a microscope slide for investigating cellular response to shear stress. The flow was controlled with an Ismatec Reglo Digital (Cole Parmer) peristaltic pump with a combination of PharMed BPT (Cole Parmer) and platinum cured silicone tubing (HelixMark) with a 1.5875 mm inner diameter. To prevent bubbles from passing through the parallel plate and disturbing the cells, a Stovall Flow Cell bubble trap was used (Fisher Scientific). All system parts were thoroughly cleaned before using with cells. The bubble trap and parallel plate were cleaned with 70% ethanol and left to dry in sterile environments, while the tubing and glass slides were autoclaved. The system
pulled medium through the bubble trap and parallel plate to help reduce unsteady flow from the peristaltic pump (Figure 38). The eight roller pump also helped reduce flow pulsatility.

Figure 38. Parallel plate flow chamber setup with all components and the flow direction indicated (Image courtesy Steve Kemeny).

The entire system was run at 37°C and 5% CO₂ in an incubator (Figure 39). Often two channels were used at the same time to reduce variation in experimental conditions.
When endothelial cells were exposed to the 20 dynes/cm² shear stress for 24 hours, they elongated and aligned in the flow direction while cells not exposed to flow maintained their polygonal shape. The actin labeled cells with or without nanoparticles showed fibers aligning the flow direction (Figure 40).
Figure 40. Images of actin filaments in cells under flow (A) static, 24 hours of flow at 20 dynes/cm² on cells not loaded with nanoparticles (C) cells loaded with 0.5 mg/ml of iron oxide nanoparticles. Scale bar is 30µm.

The angle distribution plot in Figure 41 showed magnetically labeled cells aligned more to the shear stress than the control cells. The fiber angle distribution standard deviation for static culture with nanoparticles is $52.2° ± 3.2°$ and without nanoparticles is $48.91° ± 1.1°$. Then after 24 hours of shear stress exposure, the angle distribution standard deviation for the cells loaded with nanoparticles was $32.1° ± 3.0°$ while it was $43.3° ± 3.1°$ for cells without nanoparticles. Keeping in mind that lower standard deviations mean more aligned cells, the cells with nanoparticles appeared to align more to the flow direction ($p = 0.0305$).
Figure 41. Cells loaded with nanoparticles aligned more to the 20dynes/cm$^2$ shear stress. The standard deviation of the angle distribution for the cells loaded with nanoparticles was $32.1^\circ \pm 3.0^\circ$ while it is $43.3^\circ \pm 3.1^\circ$ for cells without nanoparticles ($p=0.0305$).

These studies suggest that nanoparticle exposure changes cell response to mechanical stimuli, in this case shear stress. However these results are preliminary and should be repeated with different nanoparticle concentrations. Also a time study can also be performed whether nanoparticle uptake delays or accelerates cell response to flow. For the same nanoparticle concentrations, ROS levels in the cells should also be measured.
after 24 hours exposure to the shear stress, and the relationship between ROS formation and actin cytoskeleton alignment should be investigated.

5.2.3 Bioprinted Nanoparticles for Tissue Engineering Applications

In this thesis, we showed that a decrease in cellular viability is observed when cells were loaded with iron oxide nanoparticles and printed with the hybrid printing system. We increased the viability to some extent by decreasing the printing pressure however printing of tissue scaffolds with lower printing pressure might not be an efficient method for manufacturing since it slows down the process. For this reason, as future work, we suggest improving printed magnetically labeled cell viability by exploring different nanoparticle sizes and shapes, as well as attaching nanoparticles to the cell membrane. Bioactive factor conjugated nanoparticles can be printed with the hybrid system which would give us the ability to precisely pattern cells and bioactive factors throughout the tissue growth process and this will improve fabrication of complex tissues. We also observed that cells do not attach to alginate scaffolds which results in a decrease in cellular function and affects cell proliferation. We suggest using collagen gels with a cooled printing system. We also observed some difficulties in the nanoparticle or cell pattern movement inside the tissue scaffolds when patterns were complex shapes. To solve this problem, we suggest using complex magnet arrangements to control cell movement and patterning after printing in the tissue engineered construct.
5.3 Advancing Engineering Knowledge

Nanoparticles are an expected future manufacturing material that will make most products lighter, stronger, cleaner, less expensive and more precise. But as captivating as this technology may seem, numerous technological and market hurdles to commercialization exist. Nanoparticles can be used in industry only with a keen understanding of the basic science as applied to actual production requirements and market needs. Especially, current liquid phase synthesis methods produce low nanoparticle quantities. New techniques are needed to develop industrial viable processes which manufacture nanoparticles at high quality and large volumes. In previous research, flame operating conditions like flame temperature and configuration were shown to effect final TiO$_2$ and SiO$_2$ nanoparticle morphology and chemistry [135]. This thesis showed that iron oxide nanoparticle morphology and chemistry can also be controlled by changing flame operating conditions. These data demonstrate that critical synthesis parameters for TiO$_2$ and SiO$_2$ metal nanoparticles are also valid for iron oxide synthesis, suggesting that it might be possible to control characteristic properties of the other metal oxide nanoparticles using flame synthesis conditions as well. Additives have also previously been used to create composite metal oxide structures, such as silica coated iron oxide synthesized by Zachariah [46]. However our studies revealed that additives sometimes do not form composite structures but rather change the core synthesized metal oxide morphology while creating a heterogeneous mixture of two separate metal oxides. This implies that using additives to form composite structures should be investigated more carefully to avoid unwanted changes in final nanoparticle morphology.
We can use nanotechnology in medicine only if we can make sure that nanoparticles are not toxic to the human body. We demonstrated that as long as nanoparticle size, composition and shape are the same, the nanoparticle synthesis method does not affect cytotoxicity. This suggests that the cheapest method to produce the most accurate nanoparticles, such as flame synthesis for metal oxide nanoparticles, should be used. This thesis additionally made significant progress towards understanding nanoparticle toxicity mechanisms. We showed that reactive oxygen species formation and actin cytoskeleton disruption are the two mechanisms playing a major role in cell toxicity. ROS formation has been observed with other nanoparticles types and other cell lines. This thesis, by proving that ROS are a primary cytotoxicity mechanism for iron oxide nanoparticles and endothelial cells, further suggests that ROS generation may be a general mechanism for the nanoparticle cytotoxicity. We also demonstrated ROS blockade prevented actin cytoskeleton disruption and cell death to some extent. This finding might enable safer use of nanoparticles in medicine. If reactive species formation could be restricted after the cellular nanoparticle uptake, either by conjugating ROS blockers to the nanoparticles or by giving systemic ROS blockers before the nanoparticle application, it might be possible to reduce nanoparticle toxic side effects in the human body. In this way, higher nanoparticle doses could be used in various medical applications. Our data also suggest that ROS generated after nanoparticle uptake might also affect cell structure, cell response to mechanical stimuli, and several other key cell functions like cellular motion, intracellular transport and cellular division which depend
on the cell cytoskeleton. Scientists working with nanoparticles should also focus on
functional changes in magnetically labeled cells in addition to cytotoxicity.

Due to the accidents, birth defects and diseases, a large number of people around
the world suffer organ and tissue loss every year. Novel technologies must be developed
to enable tissue engineering of complex organs. The novel hybrid solid freeform
fabrication system enabled the fabrication of functionalized tissue scaffolds as well as
movement of cells and bioactive factors inside the tissue constructs. This expands the
capabilities of the field by making tissue engineering an active process during
development. Tissue engineers can potentially control tissue constructs after the construct
design and initial fabrication. The designed hybrid system will also make it possible to
noninvasively track the cell movement inside the constructs, which could dramatically
enhance our understanding of tissue development. The hybrid solid freeform fabrication
system has applications beyond tissue engineering. This technique will also advance
general solid freeform fabrication techniques for all-purpose manufacturing operations
since it enables layer by layer manufacture of three dimensional shapes of polymers
mixed with nanoparticles without losing the quality and geometric precision of the final
product. The decrease observed in the cell viability in magnetically labeled cells after
bioprinting revealed that cells may be more sensitive to mechanical forces when they are
loaded with nanoparticles. This should direct nanoparticle toxicity studies to changes in
cell response to mechanical stimuli when loaded with nanoparticles. Also we showed that
bioprinting effects on overall cell viability are cell type specific and for each cell line
should be investigated separately.
Overall, this thesis provided valuable insight into the synthesis of iron oxide nanoparticles, underlying toxicity mechanisms leading to cellular dysfunction and cell death and finally provided a unique tool to manufacture and noninvasively image three-dimensional tissue constructs by using principles of nanotechnology to advance the field of tissue engineering. As this work continues, I hope these discoveries will be further developed and used in medical therapies to ameliorate human disease.
LIST OF REFERENCES


VITA

EDUCATION
Ph.D., Mechanical Engineering, Drexel University, Philadelphia, 2009
M.Sc., Mechanical Engineering, Koc University, Turkey, 2004
B.Sc., Mechanical Engineering, METU, Turkey, 2002

RESEARCH EXPERIENCE
2005-2009 Research Assistant, Mechanical Engineering and Mechanics, Drexel University
Advisor: Dr Alisa Morss Clyne
2002-2004 Research Assistant, Mechanical Engineering, Koc University, Turkey
M.Sc. Dissertation: Mechanics, dynamics and thermal analysis of hard turning process

JOURNAL PAPERS

CONSULTING/MENTORING EXPERIENCE
2007-2009 Research Experience for Teachers (RET) Mentor for Drexel NSF RET summer research
2009 Drexel Mentorship Program Mentor for summer research
2006-2008 Hess Undergraduate Honors Scholar Mentor

TEACHING EXPERIENCE
2005-2009 Graduate Teaching Assistant, Drexel University
- MEM 220 Basic Fluid Mechanics, MEM 255 Introduction to Controls, MEM 361 Engineering Reliability, MEM 410 Thermodynamic Analysis
- MEM 320 Fluid Dynamics I, MEM 591 Applied Engineering Analysis Methods, Thermodynamic Analysis II, MEM 380 Introduction to Biomechanical Engineering
2002-2004 Graduate Teaching Assistant, Koc University
- Dynamics
- Mechanical Eng. Measurements and Experiments

HONORS, DISTINCTIONS, AND AWARDS
- Recipient of George Hill Jr. Fellowship, College of Engineering, Drexel University, 2008-2009
- Recipient of George Law Fellowship, College of Engineering, Drexel University, 2009-2010
- ASME Bioengineering Conference Graduate Student Travel Award, Drexel University, June 2009
- NSTI Nanotech Graduate Student Travel Award, Drexel University, June 2008
- Nominee for Drexel University Best Graduate Student Research Award, 2009