Synthesis, modification and characterization of magnetic nanoparticles and applications in reversing osteoporosis and inhibiting bacterial infection

By

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B.S., Vietnam National University, Hanoi. 2005

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  ✓ Material characterizing techniques: energy dispersive spectroscopy (EDS), X-ray diffraction (XRD), mechanical testing systems for biological samples, dynamic light scattering (DLS) and zeta-potential measurement, inductively coupled plasma mass spectrometry (ICP-MS).
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✓ Assays: cytotoxic testing (MTT and MTS assay), DAPI assay, enzyme linked immunosorbent assay (ELISA), live/dead assay (syto-9/propidium iodine), various colorimetric assays (alkaline phosphatase activity, extracellular calcium deposition, total protein synthesis, collagen synthesis, etc).

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- Materials Research Society
- Society for Biomaterials
- Vietnamese Education Foundation Fellow
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CHAPTER 1. INTRODUCTION TO ORTHOPEDIC MATERIALS AND
BIOMEDICAL APPLICATIONS OF MAGNETIC NANOPARTICLES

1.1 Bone remodeling, bone diseases and bone replacement nanomaterials

Bone and cartilage comprise the human skeletal system. Bones exist in various shapes and have complex internal and external structures which contribute to its lightweight and high strength. In the body, bone serves the following three functions [1]: (1) provides mechanical support and is the site of muscle attachment for movement; (2) protects various organs and surrounds bone marrow (a source of stem cells in the body); and (3) serves a metabolic function, storing calcium, phosphorus and other essential ions, and regulating homeostasis. While the properties and structure of bone differ depending on anatomical location, there are several commonalities between all bones since they are all natural nanostructured composite materials composed of organic compounds (mainly collagen) reinforced with inorganic compounds (hydroxyapatite).

Bone is a living tissue as it is continuously remodeled through two concurrent processes: old bone resorption by osteoclasts and new bone formation by osteoblasts [2]. The remodeling process starts with the formation and adhesion of osteoclasts to bone to begin resorption (Figure 1.1). Osteoclasts resorb bone by secreting acid and proteases. After osteoclasts complete bone resorption at a particular site, osteoblasts migrate into the resorbed site and start new bone formation by secreting osteoid (an unmineralized matrix consisting of type I collagen and other proteins). Osteoblasts express and secrete matrix proteins such as type I collagen, osteocalcin, and a key enzyme in the mineralization process, alkaline phosphatase (only active osteoblasts can produce this enzyme) [3]. The new matrix is then mineralized. While most osteoblasts migrate away from this site to
continuously create new bone (often following osteoclastic activity), some of the osteoblasts form flattened lining cells on the new bone surface, some other osteoblasts that remain in the newly mineralized matrix differentiate to become osteocytes [4]. These osteocytes are believed to be the first to respond to bone deformation or cracks. Apoptosis and deformation of osteocytes trigger lining cells and other osteocytes to release local growth factors. These factors attract cells from marrow and blood to the crack site to start osteoclastogenesis and the remodeling circle begins again [5].

Figure 1.1 Bone remodeling [5].

However, as with any organ, disease can occur and problems with bones are very common. In fact, annually, an estimated 1.5 million individuals in the United States suffer from bone fractures [6]. Among diseases leading to bone fractures, osteoporosis is the leading cause of bone fractures in both males and females at all ages. Osteoporosis is a silent disease which is characterized by reduced bone mineral density and a deteriorated structure of bone tissue [7]. Currently, some common treatment methods for osteoporosis, including anti-bone resorptive medication (such as bis-phosphonates [8-9], estrogen
therapy and hormone therapy [10]) and bone-forming medication (such as teriparatide [11]) are suggested.

A common treatment for bone fractures resulting from osteoporosis is the implantation of orthopedic prosthetics. These prostheses help heal bone non-unions and allow patients to partially regain function. Unfortunately, today’s orthopedic implant materials do not allow patients to return to their normal, daily active lifestyles they had before bone fracture. Most importantly, it has been reported that the average lifetime of an orthopedic implant is only 10 to 15 years [12]. This means that those who are young and have implants will have to undergo several additional painful and expensive surgeries to replace such failed orthopedic implants. Several factors lead to orthopedic implant failure mostly residing on the consequences of incomplete prolonged osseointegration [13-15] (i.e., a lack of bonding of an orthopedic implant to juxtaposed bone) or severe stress shielding (due to differences in mechanical properties between a metallic implant and surrounded ceramic bone [16-17]). Therefore, a great amount of effort from biomedical researchers world-wide have focused on improving the design and manufacturing of orthopedic implants to improve the bone-implant interface.

While many have attempted to alter orthopedic implant chemistry (from metals to ceramics to polymers), recent discoveries have highlighted that nanotechnology may universally improve all materials used for regrowing bone. This technology uses a wide range of nanomaterials, e.g. materials with at least one dimension from 1 – 100nm, such as metals, ceramics, polymers and composites with various structures including but not limited to particles, fibers or grains. These nanomaterials show superior properties
compared to their conventional counterparts due to their distinctive nanoscale features and novel physical properties that ensue [18-19].

Importantly, all of our tissues are nanostructured (Figure 1.2). Specifically, natural bone is composed of three levels of hierarchy [20]: (i) the nanostructure (a few nanometers to a few hundred nanometers), such as non-collagenous organic proteins, fibrillar collagen type I and embedded mineral nanocrystals; (ii) the microstructure (1 to 500 micrometers), such as lamellae, osteons and Haversian systems; and (iii) the macrostructure, such as cancellous and cortical bone. These hierarchical structures (especially at the nano level) assemble into heterogeneous and anisotropic bone and have yet to be accurately duplicated in today’s bone implants.

Figure 1.2 Representative AFM image of cortical bovine bone. Numerous nanostructured features on the surface of cortical bovine bone are visible. Root-mean-square values from AFM for 5 µm×5 µm scan was 32 nm. Adapted from [21].

Of relevance to the merging of nanotechnology and orthopedics is the realization that bone is a natural nanostructured composite material composed of inorganic
compounds (bone apatite) reinforced with organic compounds (mainly collagen) [22]; although this has been known for quite some time, only today has there been an emphasis on duplicating the same nano features in bone. Specifically, type I collagen contributes approximately 90% of the organic phase of bone and the rest (10%) consists of noncollagenous proteins and ground substances. As for the inorganic phase of bone, various forms of calcium phosphates (most notably, crystalline hydroxyapatite \(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2\) or HA) are the primary mineral component. Since bone is a hierarchical material with the lowest level belonging in the nanoscale range, materials with nanometer structures (as only nanotechnology can create) appear as natural choices for creating better bone implants.

1.2 Nanomaterials in bone related research

1.2.1 Metals

Metals have been used widely as implant materials mainly due to their superior mechanical properties which allow them to be used in load-bearing situations. They are used as bone replacement materials in hip prostheses, dental implants, etc. The most commonly used metals are stainless steel [23], cobalt chrome alloys [24], and titanium and its alloys [24-25]. Despite their advantage of mechanical properties, these materials fail after 10-15 years of implantation into the human body. Researchers explain this failure to be the consequence of incomplete osseointegration [13-15] (i.e., lack of bonding of an orthopedic implant to juxtaposed bone) or of severe stress shielding due to differences in mechanical properties between an implant and surrounded bone [16-17]. Other disadvantages of metallic orthopedic materials include the need for further operation to remove temporary implants such as plates, pins, and screws, and negative
tissue responses to the ions released from metallic implants [26-27]. However, these disadvantages common for conventional (or micron-structured) metals may not be the same for nanostructured metals.

Recently, several studies indicated increased adhesion and functions of bone cells on nanoscale surfaces of different materials including metals [18-19, 28-29], thus, providing a potential improvement for better osseointegration of orthopedic implants. For example, this research group reported increased osteoblast adhesion on nanophase metals: Ti, Ti$_6$Al$_4$V and CoCrMo compared to conventional metals [29] (with no nanoscale surface roughness) (Figure 1.3).

![SEM images of Ti, CoCrMo, and Ti6Al4V compacts. Increased nanostructured surface roughness was observed on nanophase compared to conventional Ti, CoCrMo, and Ti6Al4V. Bar=1 µm for nanophase compacts and 10 µm for conventional ones. Adapted from [29].](image)

Figure 1.3 SEM images of Ti, CoCrMo, and Ti6Al4V compacts. Increased nanostructured surface roughness was observed on nanophase compared to conventional Ti, CoCrMo, and Ti6Al4V. Bar=1 µm for nanophase compacts and 10 µm for conventional ones. Adapted from [29].

Briefly, for that study, nano and micro-size powders of Ti, Ti$_6$Al$_4$V and CoCrMo were obtained and pressed into discs before cell culture was performed and osteoblast
adhesion was observed after 1 and 3 hours. The results showed that osteoblast adhesion was significantly greater on nanophase Ti, Ti$_6$Al$_4$V and CoCrMo when compared to conventional micron sized Ti, Ti$_6$Al$_4$V and CoCrMo. It was also observed that osteoblast adhesion occurred primarily at particle boundaries. Importantly, these results indicated that increased osteoblast adhesion may be due to the greater number of defect boundaries at the surface of nanophase materials. Since adhesion of osteoblasts is a prerequisite for subsequent functions (such as deposition of calcium containing materials), this study implied further improved functions of osteoblasts on nanophase Ti, Ti$_6$Al$_4$V and CoCrMo.

A recent study by Yao et al. highlighted another way to elevate the performance of metallic materials by using the anodization process [30]. In that study, the surface of Ti can be anodized to create nanotube-like pores, which possess higher surface energy and wettability compared to unanodized Ti. This nanoscale Ti greatly improved osteoblast adhesion compared to conventional smooth surfaces [30]. Following this research direction, Sirivisoot et al. grew carbon nanotubes (CNTs) on the surface of anodized Ti to create a material for future orthopedic in situ biosensors, which could sense, detect and control bone growth [31] (Figure 1.4). The osteoblast long term function studies demonstrated significantly higher alkaline phosphate activity and calcium deposition by osteoblasts on CNTs grown on anodized Ti compared to conventional Ti as well as anodized Ti.
Figure 1.4 SEM micrographs of: (a) unanodized Ti, (b) anodized Ti without CNTs, (c) lower and (d) higher magnification of CNTs grown from the nanotubes of anodized Ti without a Co catalyst, and (e) lower and (f) higher magnification of CNTs grown from the nanotubes of anodized Ti surface with a Co catalyst [31].

Several other recent studies have indicated an improvement in bone cell functions on nanophase materials [18-19]. However, those results were ambiguous about the contribution of nanometer surface roughness since those materials also changed in surface chemistry [32-34]. Khang et al. provided additional insight about the role of
nanometer surface features towards enhancing tissue growth by comparing vascular and bone cell functions on three types of materials differing only in surface structures (i.e., flat, nanometer and sub-micron) [35]. Briefly, nanometer and sub-micron surfaces were produced via e-beam deposition of Ti, a widely used metal in orthopedics. The analysis of osteoblast adhesion on highly aligned patterns of flat/sub-micron and flat/nanometer surfaces showed that cell adhesion densities were significantly higher on both nanometer and sub-micron surfaces compared to flat surfaces. The study demonstrated that nanometer roughness had the highest efficiency for both increasing surface energy and increasing cell adhesion compared to the altered width of surface features, and, thus, suggested an important role of nanometer and sub-micron surface on the future design of improved titanium-based implant materials.

1.2.2 Ceramics

It is not just nanostructured metals that promote bone cell functions; recently, nanostructured ceramics have also been shown to promote bone cell functions compared to traditional, conventional ceramics [12, 18-19]. Ceramics have been used extensively in orthopedics due to their well known biocompatibility with bone cells and tissues [36-39]. Metallic oxides (e.g., alumina, zirconia, titania, etc.), calcium phosphates (e.g., hydroxyapatite (HA), tricalcium phosphate (TCP), calcium tetraphosphate (Ca$_4$P$_2$O$_9$)) [40] and glass ceramics (e.g., Bioglass®, Ceravital) [41] are among those commonly used in biomedical and tissue engineering applications. These ceramics are considered bioactive due to their surface properties which support bone cell adhesion, proliferation and differentiation. Specific ceramics (such as HA and TCP) have similar chemistry to the mineral phase of natural bone. Consequently, their reaction with physiological fluids
creates bonds to hard and soft tissues, thereby, increasing osseointegration between biomaterials and bone. Moreover, these ceramics are degradable and their dissolution rate depends on crystallinity. It is well known that the degradation time of amorphous calcium phosphate (8 weeks) is much shorter than that of crystalline HA (16 weeks) [42]. Therefore, they can be controlled to match the rate of new bone growth.

It has long been known that conventional ceramics (such as alumina, titania and HA with grain sizes greater than 100 nm) possess exceptional biocompatibility properties [36-39]. However, clinical applications of these ceramics still encounter many difficulties due to insufficient prolonged bonding to juxtaposed bone. In addition, applications of ceramics for large bone defects are not feasible because of the natural brittleness of ceramics. Therefore, developing novel materials that can promote and sustain osseointegration with surrounding bone over long time periods are necessary for improving implants.

The first in vitro study highlighting the influence of nanometer grain size on bone cell adhesion on ceramics was reported by Webster et al. [19]. Compared to micro-sized conventional substrates, osteoblast adhesion was significantly higher on nanophase alumina and titania substrates. Since the increase in osteoblast adhesion was observed for both nanophase alumina and titania, this study implied that the enhanced osteoblast adhesion was independent of surface chemistry and dependent only on the unique nanometer surface topography and the roughness of material; this is the same trend previously described for nanostructured metals. Further evidence of enhanced functions of osteoblasts on nanophase ceramics were also provided [18]. Osteoblast proliferation results after 5 days showed higher cell densities on nanophase titania, alumina and HA
compared to respective conventional grain size ceramics. Moreover, calcium content in the extracellular matrix of osteoblasts cultured on nanophase alumina, titania, and HA was 4, 6 and 2 times greater than on respective conventional ceramics after 28 days in culture. Overall, the results of these studies, for the first time, indicated that nanophase ceramics, with their unique surface properties, can enhance bonding of orthopedic/dental implants to juxtaposed bone and, thus, improved implant efficacy.

The mechanisms underlying the superior properties of nanophase ceramics to regenerate bone compared to conventional ones have been reported to involve vitronectin [43]. It is known that cell adhesion, an important prerequisite for further cell functions, involves proteins because osteoblast adhesion is greatly reduced in the absence of serum containing proteins regardless of ceramic grain size [44]. The results showed that vitronectin adsorbed in the highest concentration on nanophase alumina compared to conventional alumina. Decreased adsorption of apolipoprotein A-I and/or increased adsorption of calcium on nanophase alumina were possible reasons for the enhanced adsorption of vitronectin on nanophase alumina. Moreover, that study provided evidence of different conformations of vitronectin adsorbed on nanophase and conventional alumina resulting in to greater unfolding of cell-adhesive macromolecules in vitronectin on nanophase alumina to expose epitopes recognized by specific cell-membrane receptors (such as Arginine-Glycine-Aspartic acid, or RGD).

### 1.2.3 Polymers

Among all of the materials mentioned above for bone tissue engineering applications, polymers are often chosen because they have physical properties that closely resemble those of soft tissue. Polymers are easily manufactured into desirable
shapes and structures. In addition, polymers can be modified chemically or functionalized via chemical and biochemical reactions [18]. Biodegradable natural and synthetic polymers have attracted growing attention in their application as scaffold materials [45]. Biodegradable polymers can be either natural (i.e. proteins) or synthetic. In general, synthetic polymers are considered to have more advantages than natural counterparts in that they can be fabricated and tailored to give a wide range of properties and are often free from concerns of immunogenicity.

The ideal polymeric material should match the demands of a particular orthopedic applications, and, therefore, should exhibit the following properties [46]: (i) does not evoke an inflammatory/toxic response disproportionate to its beneficial effect, (ii) degrades after fulfilling its purpose and leaves no harmful components, (iii) is easily processed into the final product form, (iv) has acceptable shelf life, and (v) is easily sterilized. Controllable degradable time and matched mechanical properties are crucial since implants and scaffolds need to have sufficient strength until the surrounding tissue has been regenerated. Compared to other implant materials (such as metals and ceramics), polymers have some undeniable advantages. Their mechanical properties and degradation times can be more easily tailored than with other materials. They can also be injectable and harden in situ. In addition, a wider range of chemical properties of polymers offers a diverse number of functionalizable materials to interact with different cell types. However, the major disadvantage of using polymers is that changes in polymer formulations can eventually lead to less biocompatibility and more difficulty to sterilize compared to metals and ceramics.
The most popular natural polymer for tissue engineering is collagen. Collagen is a fibrous protein and a major component of many natural extracellular matrices. Due to its attractive biological properties (such as biocompatibility), collagen has been used for numerous scaffold fabrication processes [47-49]. However, there are still several concerns over the use of collagen for orthopedic applications because of poor handling and poor mechanical properties [20]. For applications as a scaffold, collagen has to be manufactured into a three-dimensional porous structure. This porous structure provides critical functions for the scaffold [50], such as allowing migration of cells into the scaffold, providing very large surface area for the cells to interact with the scaffold and also allowing nutrients to diffuse into the scaffold.

Most of the synthetic degradable polymers studied for orthopedic applications belong to the poly(α-hydroxy acid) family, including poly(lactic acid) (also known as polylactide – PLA), poly(glycolic acid) (also known as polyglycolide – PGA) and their copolymers such poly(lactic-co-glycolic acid) (also known as polylactide-co-glycolide – PLGA) [51-53]. These polymers are also some of the few polymers that have been approved by the FDA for human use in various medical devices. The degradation rate of these polymers can be manipulated via changes in the ratio of polylactic to polyglycolic acid, molecular weight, crystallinity, hydrophyllicity, pH of the surrounding environment, as well as specimen size, geometry, porosity, surface properties and sterilization process. For example, in the same conditions, hydrophilic PGA degrades much faster than the hydrophobic PLA in vivo or in aqueous solutions. Therefore, PGA is frequently used as a component to fabricate a copolymer with PLA to control the degradation rate of the copolymer.
Just like the ceramics and metals mentioned above, several studies have indicated that the efficacy of polymeric materials for bone regeneration can be improved through the use of nanotechnology. Recent research results have indeed demonstrated that the adsorption and conformation of proteins (such as fibronectin and vitronectin), which regulate osteoblast adhesion and functions, were enhanced on nanophase materials [14, 44]. Wei et al. reported the selective adsorption of proteins, including fibronectin and vitronectin, on 3D nanofibrous scaffolds [54]. In that study, nano hydroxyapatite/poly(L-lactic acid) (NHAP/PLLA) composite scaffolds were prepared using phase separation techniques and compared to micro hydroxyapatite/poly(L-lactic acid) (MHAP/PLLA) scaffolds. The results showed significantly greater protein adsorption on NHAP/PLLA scaffolds with higher HA content. Consequently, this led to enhanced osteoblast functions on nanofibrous polymer scaffolds. In Figure 1.5, PLGA was chemically treated in NaOH 0.1 N and HNO$_3$ 10 N for various periods of time to obtain polymer nanostructures [55].

![Figure 1.5 SEM images of chemically treated PLGA surfaces. Representative scanning electron micrograph images of (a) chemically untreated (conventional) PLGA (feature dimensions 10,000–15,000 nm) and (b) chemically treated nano-structured PLGA (feature dimensions 50–100 nm). Scale bar=100,000 nm. Adapted from [55].](image-url)
The mechanism of increased adsorption of selected proteins (such as fibronectin and vitronectin) on polymeric nanophase materials can be explained as the result of their surface energetic changes compared to conventional materials [43-44]. It is envisioned that one day, nanotechnology will create the specific surface energy required to promote the adsorption of a select protein important for mediating the adhesion of a certain cell, promising future directions for nanotechnology already underway.

Surface roughness is a critical difference between nanophase and conventional materials. Previous studies on nanometer materials showed that merely by changing the surface roughness, one can enhance osteoblast adhesion and functions such as proliferation, alkaline phosphatase activity and calcium deposition [29-30]. Recently, Price et al. [56] conducted a study of osteoblast behavior on polymer casts of consolidated carbon nanofiber-based materials, which have previously been shown to improve cell functions compared to conventional carbon fiber materials [57]. They demonstrated increased osteoblast adhesion on polymer casts of nanophase carbon fibers compared with polymer casts of the conventional carbon fibers. Similarly, polymer casts of composites of polycarbonate urethane/carbon nanotubes also promoted osteoblast functions compared with casts of polycarbonate urethane/conventional carbon tubes [58]. All of this evidence once again signifies the important role of nanoscale surface structures for bone applications. These results also provided great excitement concerning a new class of materials using nanotechnology and combining advantages of both ceramics and polymers into nanocomposites.
1.2.4 **Ceramics/Polymer nanocomposites**

Previously reported promising results of nanophase ceramics and polymers independently led to the idea of combining advantages of both types of materials to create better bone scaffold materials [59-61]. For example, scaffold materials such as hydroxyapatite (HA) can facilitate greater osteoconduction and bone cell functions than conventional materials [62]. However, it is not osteoinductive and its biodegradation rate is too slow. To avoid the drawback, biodegradable polymers can be employed to make a composite in conjunction with osteogenic potential cells and osteoinductive growth factors [63]. The more important point is that the nanocomposites can mimic the constituent features of natural bone to some extent [64]. This is because the natural bone is in fact a composite material composed of organic compounds (a polymer-like matrix, mainly collagen) reinforced with inorganic compounds (ceramic particles such as calcium phosphate derivatives). Recent studies suggested that better osteoconductivity can be achieved by synthetic materials which resemble the size and morphology of the inorganic particles of bone [65-66]. Bone cell functions can be enhanced by interacting with nanophase bioactive ceramics [67-71]. For example, Jung et al. conducted research on PLA/calcium metaphosphate composites which showed that osteoblast functions on the composite were significantly enhanced compared to scaffolds made of PLA only [72].

The ceramic materials chosen for composite fabrication are often calcium phosphates (i.e. HA), metal oxide (i.e. titania, alumina) and glass ceramics (Bioglass®). The choices for polymer materials usually are collagen (a sort of natural polymer) or from poly(α-hydroxy acid) family (synthetic polymer) including (PLA, PGA and their copolymer PLGA). For example, Du et al. developed nano-HA/collagen composite
scaffolds which promoted the deposition of a new bone matrix at the interface of bone fragments and the composite. Furthermore, they also demonstrated that the porous nano-HA/collagen scaffold provided a microenvironment resembling that seen in vivo, and osteoblasts within the composite eventually acquired a three-dimensional polygonal shape [66]. Some more recent developments of nanocomposite research were presented by Webster et al. [64, 73]. They reported nanocomposites of titania/PLGA prepared with different ultrasonic powers to ensure dispersion of nanoceramic particles (Figure 1.6). The study demonstrated that osteoblast adhesion and functions increased on nanocomposites at all sonication powers compared to pure PLGA scaffolds. Among those composites, the one that had the closest surface roughness to natural bone showed the greatest cell adhesion and subsequent calcium containing mineral deposition [63]. Up to three times more osteoblasts adhered onto nanophase titania/PLGA composites than conventional titania at the same weight ratio and porosity [73].
Figure 1.6 AFM images of nanophase titania and the PLGA mold of nanophase titania, conventional titania and the PLGA mold of conventional titania. Images provided evidence of the successful transfer of the surface roughness of nanophase titania to PLGA molds of nanophase titania, and conventional titania to PLGA molds of conventional titania. Root-mean-square values from AFM for nanophase titania at $5 \, \mu m \times 5 \, \mu m$ and $25 \, \mu m \times 25 \, \mu m$ scans were 29 and 22 nm, respectively. Root-mean-square values from AFM for the PLGA mold of nanophase titania at $5 \, \mu m \times 5 \, \mu m$ and $25 \, \mu m \times 25 \, \mu m$ scans were 35 and 27 nm, respectively. Root-mean-square values from AFM for conventional titania at $5 \, \mu m \times 5 \, \mu m$ and $25 \, \mu m \times 25 \, \mu m$ scans were 12 and 11 nm, respectively. Root-mean-square values from AFM for the PLGA mold of conventional titania at $5 \, \mu m \times 5 \, \mu m$ and $25 \, \mu m \times 25 \, \mu m$ scans were 13 and 12 nm, respectively. (a) Nanophase titania. (b) PLGA mold of nanophase titania. (c) Conventional titania. (d) PLGA mold of conventional titania. Adapted from [21].

Another advantage of nanocomposites compared to conventional materials is their great improvement in mechanical properties over polymers and ceramics. For instance, bioactive ceramics are known for their biocompatibility with bone cells and tissues [36-39], however, ceramic materials are too brittle for orthopedic applications. By combining with a polymer, nanoceramics can be manufactured to possess hardness, bending,
compressive and tensile strengths similar to those of natural bone. In fact, McManus et al. reported greater mechanical properties of polymer composites when nanometer ceramic grain sizes were added [74]. Composites of PLA with 40 and 50 wt % nanophase (<100 nm) alumina, titania, and HA showed significantly greater bending moduli than that of composites with conventional coarse (or micron sized) grain ceramics. Specifically, the bending modulus of nanophase titania/PLA composites with a weight ratio of 50/50 was 1960 ± 250 MPa, which was on the same order of magnitude to trabecular bone. On the other hand, the bending modulus of plain PLA and conventional titania/PLA samples with the same weight ratio was only 60 ± 3 MPa and 870 ± 30 MPa, respectively [74].

Nanocomposites, though, have not yet been fully explored for orthopedic application. Their enhanced mechanical and biocompatibility properties certainly provide great promise for future bone applications.

In the next part of the chapter, another promising area in nanotechnology, magnetic nanoparticles, will be presented.

1.3 Magnetic nanoparticle research and applications

In recent years, nanometer particles have drawn a great deal of interest from the biomedical research world. Nanoparticles with sizes less than 100 nm possess unique properties (such as high surface-volume ratios, high reactivity, etc.) compared to their bulk micron-structured counterparts due mainly to size effects and surface phenomena at the nanoscale [75]. Magnetic nanoparticles are particularly promising for several biomedical applications, such as: (a) cellular therapy involving cell labeling and targeting and as a tool for cell-biology research to separate and purify cell populations [76-77]; (b)
tissue repair [78-80]; (c) targeted drug delivery [81-83]; (d) magnetic resonance imaging (MRI) [84-85]; (e) hyperthermia for cancer treatment [86-89]; etc.

For more effective therapeutic treatments, materials with high saturation magnetization (such as transition metals (e.g. Fe, Co, Ni) or metal oxides (e.g. Fe$_3$O$_4$, $\gamma$-Fe$_2$O$_3$)) are usually considered. Although pure metals possess the highest saturation magnetization, they are highly toxic and extremely sensitive to oxidation [90], therefore, without a further appropriate surface treatment, such pure metal nanoparticles are not relevant for biomedical applications [91-93]. In contrast, iron oxides are less sensitive to oxidation and, therefore, can provide a stable magnetic response. In fact, small iron oxide nanoparticles have been used for in vitro diagnosis for about 50 years [94]. Recent studies have demonstrated that magnetite (Fe$_3$O$_4$) and maghemite ($\gamma$-Fe$_2$O$_3$) are very promising candidates due to their biocompatibility and relative ease to functionalize (for example with polymers such as dextrant [95-98], polyethylene glycol (PEG) [99-101], polyvinyl alcohol (PVA) [102-104] or functional groups such as thiols, amines and carboxylic acids [105]) for a wide range of applications [106-110].

For practical purposes, these nanoparticle surfaces must be tailored to improve biocompatibility properties and reduce aggregation. Without any surface modification, magnetic iron oxide nanoparticles possessing hydrophobic surfaces with large surface area to volume ratios tend to agglomerate and form larger clusters, resulting in increased particle sizes [111]. These agglomerations have strong dipole-dipole interactions and ferromagnetic behavior [112]. Clusters will be further magnetized in a magnetic field, causing stronger attraction between the magnetic nanoparticles and consequently, creating increased aggregation. Moreover, nanoparticles with proper surface coatings to
avoid such agglomerations can stay longer in circulation and are less recognized by the body’s own biological particulate filters, the reticulo-endothelial system (RES) [113]. Magnetic nanoparticles can be functionalized with organic materials (e.g., polymers such as dextrant [114] and polyethylene glycol (PEG) [115-116]) or inorganic metallic (e.g., gold [117]) or oxide (e.g., silica or alumina [118]) materials to achieve desirable dispersibility. In all cases, magnetic nanoparticles less than 15 nm in diameter are of interest because they exhibit superparamagnetic properties, meaning that they do not retain any magnetism after removal of a magnetic field and can avoid the RES [111].

Iron oxide magnetic nanoparticles can be prepared by several different methods including co-precipitation, thermal decomposition and/or reduction, micelle synthesis, hydrothermal synthesis, and laser pyrolysis techniques. A more extensive review can be found in the work by Tartaj et al. [119] and Lu et al. [120]. A brief summary is shown in Table 1.1.

Table 1.1 A summary comparison of various magnetic nanoparticle synthesis methods [120].

<table>
<thead>
<tr>
<th>Synthetic method</th>
<th>Synthesis</th>
<th>Reaction temp. [°C]</th>
<th>Reaction period</th>
<th>Solvent</th>
<th>Surface-capping agents</th>
<th>Size distribution</th>
<th>Shape control</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>co-precipitation</td>
<td>very simple, ambient conditions</td>
<td>20-90</td>
<td>minutes</td>
<td>water</td>
<td>needed, added during or after reaction</td>
<td>relatively narrow</td>
<td>not good</td>
<td>high/ scalable</td>
</tr>
<tr>
<td>thermal decomposition</td>
<td>complicated, inert atmosphere</td>
<td>100-320</td>
<td>hours days</td>
<td>organic compound</td>
<td>needed, added during reaction</td>
<td>very narrow</td>
<td>very good</td>
<td>high/ scalable</td>
</tr>
<tr>
<td>microemulsion</td>
<td>complicated, ambient conditions</td>
<td>20-50</td>
<td>hours</td>
<td>organic compound</td>
<td>needed, added during reaction</td>
<td>relatively narrow</td>
<td>good</td>
<td>low</td>
</tr>
<tr>
<td>hydrothermal synthesis</td>
<td>simple, high pressure</td>
<td>220</td>
<td>hours ca. cays</td>
<td>water-ethanol</td>
<td>needed, added during reaction</td>
<td>very narrow</td>
<td>very good</td>
<td>medium</td>
</tr>
</tbody>
</table>

The aim of this next sections is to provide information specially regarding nanoparticles in orthopedic research, new applications of magnetic nanoparticles in
biomedical research as well as the challenges they have to overcome before experiencing wide spread clinical use.

1.4 Applications of magnetic nanoparticles in biomedical research

1.4.1 Drug delivery

One of the most desirable applications for magnetic nanoparticles is targeted drug delivery to fight cancer [121-122], where nanoparticles are functionalized with appropriate groups (such as (poly(methyl methacrylate) or PMMA [123], PEG [124], etc.) loaded with drugs (carboplatin [123], doxorubicin [124], paclitaxel [125], 5-fluorouracil [126], epirubicin [127], etc.) and directed to and focused at tumor sites by an external magnetic field. For these applications, the size, surface chemistry, and charge are particularly important to ensure that the nanoparticles can stay for a long time in circulation. It is believed that magnetic nanoparticles with sizes ranging from 10 to 100 nm are most suitable for drug delivery applications [128]. The lower threshold is based on the fact that particles smaller than 10 nm are easily removed by extravasation and renal clearance [129]. The upper threshold is not well defined, however, some recent data suggest that nanoparticles 50-100 nm are smaller than the spleen cut off (200 nm) and can penetrate into large tumors following systemic administration [130]. It is well known that a magnetic nanoparticle hydrophilic coating (such as PEG or monosialoganglioside) can enhance the ability of nanoparticles to evade the reticuloendothelial system (RES) and, thus, improve circulation time in vivo for up to 6 hours after injection [131-132]. Hydrophilic nanoparticles, such as polyvinylpyrrolidone (PVP), evade the RES nearly 100% [133].
Importantly, though, magnetic nanoparticle based drug delivery systems are not new and have been developed since the 1970s. For example, in 1976, Zimmermann and Pilwat [134] used magnetic erythrocytes for the delivery of a cytotoxic drug, methotrexate. In the 1980s, several authors developed delivery strategies for various drugs using microcapsules and microspheres [135-136]. The first animal study using magnetic nanoparticle drug delivery was conducted by Lubbe et al. [137], in which small amounts of the ferrofluid were used as vehicles to concentrate epirubicin locally in tumors. The study concluded that the ferrofluid did not cause any major abnormalities and there was no LD$_{50}$. Therefore, the magnetic fluid was termed safe and can be used for cancer treatment. The results led to Phase I and Phase II clinical trials by the same research group in 1996 and 2001 [137-138]. The results demonstrated that magnetic drug targeting with epirubicin was well tolerated by patients and that the nanoparticles were successfully directed to the tumors in about one-half of the patients. More recently, several groups reported successful cytotoxic delivery and tumor remission in several animal models including swines [139], rabbits [140-141], and rats [142].

1.4.2 Hyperthermia

Hyperthermia is a method of using heat as a treatment for cancer [143]. Fundamentals of hyperthermia are based on the fact that cells (cancer and healthy cells) show signs of apoptosis when heated in the range of 41°C to 47°C [143-145] and necrosis when heated to above 50°C. Moreover, tumor cells are considered more susceptible to heat than normal cells due to their higher rate of metabolism [146] which makes hyperthermia a very promising cancer treatment.
Hyperthermia can be generated by radio frequency, microwave and laser wavelengths, but magnetic nanoparticle based heating is superior due to the following reasons: (a) it provides a non-invasive way to raise cell temperatures to a therapeutic level; (b) magnetic nanoparticles can be visualized using MRI, thus, combining diagnostic and therapeutic approaches in one type of particle; and (c) the particles can also be functionalized and combined with other types of treatment such as chemotherapy or radiotherapy. In general, the steps taken for magnetic nanoparticle hyperthermia involve the delivery of particles into tumors and heating of the particles by using alternating magnetic fields to achieve desired temperatures [147]. The heating mechanism of magnetic nanoparticles is based on Brown relaxation (i.e., heat due to friction arising from total particle oscillations) and Neél relaxation (i.e., heat due to rotation of the magnetic moment with each field oscillation) [148]. It is necessary to note that this heating mechanism is not limited to magnetic nanoparticles but is also applicable for other materials with near infra-red (NIR) absorption capabilities, such as gold nanoparticles [149-150] or carbon nanotubes [151].

There have been many publications concerning the use of magnetic nanoparticles as hyperthermia agents \textit{in vitro} and in small animal models [87, 152-154]. However, a clinical breakthrough was only made in 2007 when Maier-Hauff et al. studied therapeutic hyperthermia induced by heating implanted magnetic nanoparticles [155]. In that study, fourteen patients with recurrent glioblastoma multiforme, a type of severe brain cancer, received an intratumoral injection of aminosilane coated iron oxide nanoparticles. The tumor sites were located by several comprehensive MRI scans. The patients were then exposed to an alternating magnetic field to induce particle heating. The
superparamagnetic iron oxide nanoparticles with core sizes of 15 nm were dispersed in water at an iron concentration of 112 mg/ml. 0.1 ml to 0.7 ml of the nanoparticle solution per ml of tumor was injected into each tumor and exposed to a magnetic field of 3.8 to 13.5 k/Am alternating at 100 kHz. The authors demonstrated that all patients tolerated the nanoparticles without any complications. The T$_{90}$ median temperature (i.e., the temperature achieved by 90% of the measured tumor sites) was 40.5°C and was effectively controlled. Follow-up CT scans and reproducible temperature measurements showed that the nanoparticle deposits were stable for several weeks (Figure 1.7).

![Image](image.png)

**Figure 1.7** Three-dimensional reconstruction image (MagForce NanoPlan® software) of a skull with a frontal glioblastoma multiforme after magnetic resonance imaging and computed tomography. A calculated 42°C treatment isotherm surface (transparently red) enclosing the whole tumor (brown), thermometry catheter (green), and ventricle (light blue) are shown. Reprinted with permission from [155].
The same group of researchers also started clinical studies on hyperthermic nanoparticles to treat prostate cancer [88-89]. They also formed a company, MagForce Nanotechnologies AG, trying to commercialize the iron oxide based magnetic nanoparticles (NanoTherm® and NanoPlan®). A further review concerning the progress of the application of magnetic nanoparticles as hyperthermia agents can be found in [156].

1.4.3 Tissue repair

A new idea of using magnetic nanoparticles for cell therapy originally used magnetic labeling to track or monitor cell migration in vivo by MRI [157]. In such cellular therapeutic applications, cells were loaded with magnetic nanoparticles, directed and focused by external magnetic fields to desired sites for tissue repair. Several cell types including erythrocytes, natural killer cells and mesenchymal stem cells (MSC) have been used to test this strategy. For example, natural killer cells were magnetically labeled and directed by a magnetic field toward human osteosarcoma cells in vitro to treat bone cancer [158]. Since then, efforts have been made to apply this physical targeting strategy in vivo. Arbab et al. [159] injected magnetically labeled MSCs intravenously through the tail vein of rats and used an external magnetic field to retain cells in the liver. The results showed significantly higher numbers of labeled cells in the animal group exposed to the magnetic field compared to the group without an external magnetic field. A similar method was also used to deliver labeled endothelial cells to the surface of a steel stent [160]. Encouraging results ensued to support the tremendous potentials of cell therapies using magnetic nanoparticles, however, significant efforts are needed especially in
targeting and controlling superparamagnetic nanoparticles in the body using an external magnetic field.

Despite all of these promising results towards treating cancer and for various cell therapies, the use of iron oxide nanoparticles in orthopedic applications remains largely unexplored. Pareta et al. were the first to use magnetic nanoparticles in an effort to reverse osteoporosis [79]. The general idea was to fabricate and modify magnetic nanoparticles with surfactants and drug coatings before injection into porous bone sites. The drug coated magnetic nanoparticles can then be directed through the intricate bone structures by an external magnetic field. Eventually, after the magnetic field is removed, the magnetic nanoparticles can attach to osteoporotic bone, immediately build bone mass while promoting new bone growth.

Specifically, in that study, iron oxide nanoparticles (Fe$_3$O$_4$ and γ-Fe$_2$O$_3$) were prepared via a wet chemistry method under high pH. The particles were further tailored with a calcium phosphate coating to treat osteoporosis. Bovine serum albumin (BSA) and citric acid (CA) were used as surfactants to prevent nanoparticle agglomeration. These nanoparticle solutions were later added into osteoblast culture media and incubated with the cell for 1 day. The study showed for the first time significantly increased osteoblast density in culture wells in the presence of BSA and iron oxide nanoparticles compared to the controls (without any nanoparticles). Furthermore, the results also demonstrated greater osteoblast densities in the presence of maghemite nanoparticles than magnetite nanoparticles and controls after 5 and 8 days. While the mechanism remains unclear, one proposed explanation relates to the adsorption of specific proteins known to promote bone cell functions (such as vitronectin and fibronectin) on nanoparticle surfaces.
Vitronectin is a protein known for promoting osteoblast adhesion, a prerequisite for subsequent cell functions. Previous evidence also demonstrated greater vitronectin adsorption on nanophase surfaces than on conventional larger size micron surfaces [43]. Therefore, fabricating nanoparticles that do not agglomerate and promote vitronectin adsorption might be essential for promoting osteoblast functions. Clearly, this study exhibited potential in treating not only osteoporosis but also other local bone diseases and fractures. However, further evidence is needed to confirm enhanced osteoblast functions in the presence of iron oxide nanoparticles and \textit{in vivo} verification of such promising \textit{in vitro} results.

1.4.4 Infection

Fighting against bacterial infection has always been crucial for any biomedical application. It has been known for quite a while that bacteria can adhere to solid surfaces of biomedical instruments, catheters, and implants, creating biofilms leading to many infectious diseases [161]. As an indication of our failed attempts so far, an increasing number of bacteria have built resistance to conventional antibiotics (such as ampicillin, ciprofloxacin [162], cloxacillin [163], methicillin [164], and penicillin [165]). Many believe that using magnetic nanoparticles to carry antibacterial agents (TiO$_2$, ZnO, MgO, chitosan, copper, silver, etc.) [166-174] can provide an alternative treatment method not based on antibiotics to fight bacterial infections [175]. For example, Lee et al. synthesized magnetic beads coated with silver (Ag) to inhibit \textit{Escherichia coli} (\textit{E. coli}) colonization [176]. These microspheres were localized by an external magnetic field showing clear antibacterial activity in the focused zone (Figure 1.8). By reducing the magnetic particle size to the nano-scale, it is expected that the consequences of higher
surface – volume ratios can lead to greater, larger amounts of loaded antimicrobial drugs, thus, providing a more effective treatment.

Figure 1.8 Localized delivery of multilayer coated magnetic microparticles by magnetic fields: (a) scheme of the experimental setup and (b) fluorescence microscope images at locations (i), (ii), and (iii) in the Petri dish. Live and dead *E. coli* were stained with green and red fluorescence dyes, respectively. The distances from (i) to (ii) and (iii) were ~ 3 and ~ 15 mm, respectively. The scale width of each image is 60 µm. Reprinted with permission from [176].

1.5 Future challenges

Nanotechnology has developed to an extent that makes it possible to fabricate improved materials with more favorable properties for bone applications. However, it should be noted that studies on nanophase materials have only just began; there are still many unanswered questions and unexplored areas which can greatly improve not only the efficacy of the nanotechnology based bone implants, scaffolds and drug delivery systems but also our understanding of the mechanisms of nanoparticle recognition by cells. The challenges that are needed to overcome for creating a better material for bone applications are addressed here.
1.5.1 Toxicity of nanoparticles

It has been reported that over 500 consumer products contain nanoscience and nanotechnology related materials [177]. Currently, there are at least 12 nanomedicines already approved by the FDA and many more are in their final development stages [177]. Clearly, the toxicity of magnetic nanoparticles is one of the most important issues that needs further investigation. It has been difficult to report accurate nanoparticle toxicity since toxicity depends on numerous factors including dose, chemical composition, method of administration, size, biodegradability, solubility, pharmacokinetics, biodistribution, surface chemistry, shape and structure, to name but a few [75]. Among these factors, modification of magnetic nanoparticle surface properties can be a main tool to reduce toxicity [178].

To fully understand the toxicity of nanoparticles, extensive cytotoxicity studies should be conducted not only in vitro but also in vivo since the in vitro experimental results can be misleading [179]. Importantly, it has been reported that some nanomaterials interfere with cell viability assays [180]. For example, Monteiro-Riviere et al. recently demonstrated that classical dye-based assays (such as MTT and neutral red (NR) assays that determine cell viability) produce invalid results with some nanomaterials due to nanoparticle/dye interactions [181]. Therefore, several different assays would be necessary to confirm the toxicity of nanoparticles [182].

Regarding the toxicity of magnetic nanoparticles, the most widely studied nanoparticles are iron oxide nanoparticles. These nanoparticles (such as Feridex®, Endorem®, etc.) have been widely used in MRI applications and are considered biocompatible. Cengelli et al. reported that iron oxide nanoparticles coated with dextran
and various PVAs were not cytotoxic to brain-derived endothelial EC219 cells and murine N9 and N11 microglial cells [183]. Müller et al. demonstrated that the iron oxide nanoparticle Ferumoxtran-10 (Sinerem®, Guerbet, France, Combidex®, Advanced Magnetics, USA) had no toxic effects for human monocyte-macrophage interactions at concentrations up to 1 mg/ml over 72 h and may be only slightly toxic at the extremely high concentrations of 10 mg/ml [184].

1.5.2 Targeting and controlling magnetic nanoparticles by an external magnetic field

One of the largest challenges in magnetic particle based therapeutics is the ability to direct the magnetic nanoparticle drug carriers to the desired site for treatment. Many efforts have been employed to develop magnetic carriers; however, control systems for these nanoparticles are still very simple, mostly consisting of just a permanent magnet placed near the target site [127, 137, 185-186]. Using this control system is not ideal for particle focusing, and moreover, the magnetic field from permanent magnets can only penetrate into a tissue depth of 8-12 cm [187-188], thus, this strategy cannot be applied for deeper tumors. One way to enhance local drug delivery is by using magnetic implants which can attract magnetic nanoparticles if needed [189]. Shapiro also suggested using dynamically controlled magnetic fields to focus magnetic carriers to deep tissue targets [190]. A magnetic control model consisting of 8 magnets was also developed where each magnet was controlled independently using a special algorithm. The modeling results demonstrated that dynamically controlled magnets could drive a magnetic fluid through the center and create a hot spot at the target (Figure 1.9). Although this method seemed very promising, it is necessary to note that controlling nanoparticles in vivo is significantly more complicated. There are some available models regarding this matter.
[191-193], but an optimized algorithm incorporating complex vasculature geometry and blood flow still needs to be developed.

Figure 1.9 Modeling results of a dynamic control magnet system. (A): Constant actuation by a single magnet on the far left; (B): Dynamic control was achieved by turning a magnet on and off to drive the ferrofluid toward the center; and (C): A time-
averaged ferrofluid concentration map showing the hot spot at the center. Reprinted with permission from ref [190].

1.6 Commercialization and conclusions

In summary, there is an exciting amount of evidence that strengthens the belief that nanometer particles can improve disease prevention, diagnosis, and treatment; especially new studies are continuously highlighting the role that magnetic nanoparticles may play in reducing infection and promoting bone growth. The future of magnetic nanoparticle applications involves the creation of multifunctional therapeutic materials [107-108, 117, 151] and the ability to target those nanoparticles to desirable sites. Many products based on magnetic nanoparticles are in their final development stages with several already on the market (Table 1.2). With new knowledge gained concerning how the human body interacts with magnetic nanoparticles (from killing cancer cells to healing tissues to reducing infection), advanced applications of magnetic nanoparticles for treating a wide range of diseases may be available in the very near future.

In the next chapters, the design, fabrication and testing of magnetic nanoparticles with hydroxyapatite coatings are discussed in the context of osteoporosis treatment.
Table 1.2 Examples and properties of commercial magnetic iron oxide nanoparticles. Adapted and updated from [114, 194]. SPIO: superparamagnetic iron oxide (300 nm - 3.5 µm diameters); SSPIO: standard SPIO (60-150 nm diameters); USPIO: ultrasmall SPIO (10-40 nm diameters).

<table>
<thead>
<tr>
<th>Agents</th>
<th>Class</th>
<th>Trade and common names</th>
<th>Applications</th>
<th>Status</th>
<th>Mean particle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMI-121</td>
<td>Oral SPIO</td>
<td>Lumirem®, Gastromark®, Ferumoxsil®</td>
<td>MRI contrast agent for bowel imaging</td>
<td>Approved in EU, South America, and Southeast Asia.</td>
<td>&gt;300 nm</td>
</tr>
<tr>
<td>OMP</td>
<td>Oral SPIO</td>
<td>Abdoscan®</td>
<td>MRI contrast agent for bowel imaging</td>
<td>Approved in EU but discontinued.</td>
<td>3.5 µm</td>
</tr>
<tr>
<td>AMI-25</td>
<td>SSPIO</td>
<td>Feridex®, Endorem®, Ferumoxide®</td>
<td>MRI contrast agent for liver imaging</td>
<td>Approved in EU.</td>
<td>80-150 nm</td>
</tr>
<tr>
<td>SHU555A</td>
<td>SSPIO</td>
<td>Resovist®</td>
<td>MRI contrast agent for liver imaging</td>
<td>Phase III (USA) Approved (Japan, EU, Australia)</td>
<td>62 nm</td>
</tr>
<tr>
<td>AMI-227</td>
<td>USPIO</td>
<td>Sinerem®, Combidex®, Ferumoxtran®</td>
<td>MRI contrast agent for metastatic detection in lymph nodes</td>
<td>Phase III</td>
<td>20-40 nm</td>
</tr>
<tr>
<td>CODE 7228</td>
<td>USPIO</td>
<td>Feraheme® (ferumoxytol)</td>
<td>Treatment of iron deficiency anemia; MRI contrast agent for vascular imaging</td>
<td>Phase II</td>
<td>18-20 nm</td>
</tr>
</tbody>
</table>
CHAPTER 2. SYNTHESIS OF HA COATED AND UNCOATED MAGNETIC NANOPARTICLES AND THEIR INFLUENCE ON OSTEOBLAST PROLIFERATION AND FUNCTION

2.1 Introduction

2.1.1 Osteoporosis, current treatments and disadvantages

Osteoporosis is a very common bone disease that causes reduced bone mass leading to an increased risk of bone fractures. Statistics show that approximately 44 million Americans are affected by osteoporosis [195]. While 10 million people have been diagnosed with the disease, 34 million people are suffering from low bone mass and are considered at an increased risk for osteoporosis [195]. Having osteoporosis can affect patients not only in terms of health but also financially. In 2005, it was estimated that osteoporosis-related bone fractures collectively cost $19 billion U.S. dollars [195]. Therefore, finding a new and better way to treat osteoporosis is of great interest clinically as well as for society as a whole.

Although there is no cure for osteoporosis, lifestyles and calcium or nutritional supplements can help towards prevention. For more severe cases, medication is needed. Currently, some common treatment methods for osteoporosis, including anti-bone resorptive medication (such as bis-phosphonates [8-9], estrogen therapy and hormone therapy [10]) and bone-forming medication (such as teriparatide [11]) are suggested. Anti-resorptive drugs have been used to further reduce the risk of bone fractures by inhibiting the activity of osteoclasts (bone resorbing cells). However, drugs of this type (such as bis-phosphonates) can have non-specific effects on other cell types. For instance,
it has been shown that bis-phosphonates decrease osteoblast (bone forming cells) proliferation and induced macrophage (one type of white blood cells) apoptosis [196-197]. Another limitation of these treatments is the inability to induce new bone growth but rather focus on stopping bone resorption. Anabolic medication (such as teriparatide) can effectively build bone mass, but such drugs also have several serious side effects and induce non-specific bone growth in undesirable regions (such as the liver). Moreover, many of these treatments are taken orally, thus, bioavailability is reduced. More details about current medication treatments and disadvantages are presented in Table 2.1.

For the above reasons, a site specific drug delivery system, which can induce osteoblast growth directly at the site of low bone mass, is believed to be more effective in treating osteoporosis than systemically delivered drugs.

Table 2.1 Current common osteoporosis treatments, mechanisms and disadvantages.

<table>
<thead>
<tr>
<th>Osteoporosis treatments</th>
<th>Primary mechanisms</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td>Bisphosphonates (leendronate (Fosamax), ibandronate (Boniva), risedronate (Actonel) and zoledronic acid (Reclast)).</td>
<td>Inhibits the digestion of bone by encouraging osteoclasts to undergo apoptosis, or cell death, thereby slowing bone loss [9].</td>
<td>Many side effects, some severe including: nausea, abdominal pain, difficulty swallowing and the risk of an inflamed esophagus or esophageal ulcer. More serious side effects with bisphosphonates: such as osteonecrosis of the jaw, a rare type of thigh fracture, irregular heartbeats and visual disturbances.</td>
</tr>
<tr>
<td>Hormone therapy [198-</td>
<td>Estrogen can control osteoclast</td>
<td>Can increase the risk of blood</td>
</tr>
<tr>
<td>199] (Estrogen).</td>
<td>apoptosis and, thus, inhibits bone resorption [200].</td>
<td>clots, endometrial cancer, breast cancer and possibly heart disease [201].</td>
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<tr>
<td>Selective estrogen receptor modulators (SERMs) such as Raloxifene (Evista) [202-203] and Lasofoxifene [204-205].</td>
<td>Inhibition of osteoclastogenesis and inhibition of osteoclast activity [10, 206].</td>
<td>Side effects: Uterine cancer, hot flashes, venous thrombosis [206]. Cannot be used for men.</td>
</tr>
<tr>
<td>Calcitonin (Miacalcin, Fortical).</td>
<td>Calcitonin reduces bone resorption [207] and may slow bone loss by decreasing osteoclast formation [208] and osteoclast attachment [209].</td>
<td>Nasal irritation, nose bleeding, nausea and hot flashes [210-211]. Not as potent as bisphosphonates.</td>
</tr>
<tr>
<td>Teriparatide (Forteo).</td>
<td>Teriparatide is the portion of the human parathyroid hormone, which regulates calcium and phosphate metabolism in bone [11, 212]. Teriparatide stimulates new bone growth by increasing osteoblastic differentiation and decreasing osteoblastic apoptosis [213].</td>
<td>Side effects: dizziness, leg cramps. Some patients developed acute gout [11], higher risk of osteosarcoma [214].</td>
</tr>
</tbody>
</table>

### 2.1.2 Potential osteoporosis treatments using a novel drug delivery system

In this study, a brand new direction was taken to produce a novel magnetically-driven nanoparticle based therapeutic agent which may provide a more efficient and direct treatment for bone defects, such as osteoporosis.
Hydroxyapatite (HA) is a material very similar to the mineral component of bone. It is well established that osteoblasts grow better on HA coated metals than metals alone [19]. By creating nanometer surface roughness features on HA, one can further enhance osteoblast adhesion, proliferation and differentiation, to provide quicker bone regeneration [18]. Research has shown that nano surface features enhance the adsorption of vitronectin and fibronectin (proteins that are favorable for osteoblast adhesion) and thus increase new bone growth [215]. HA has also been tested clinically to prevent osteoporosis [216-217]. Specially, patients with osteoporosis were treated with HA containing compounds orally (other components in the compounds were collagen type I and non-collagenous proteins/peptides containing insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), transforming growth factor beta (TFG-β) and osteocalcin). The results showed that HA compounds more effectively slowed bone loss compared to untreated patients [216] or calcium carbonate treated patients [217]. However, these treatments had significant drawbacks because they increased non-specific bone formation.

In the current study, the combination of the success of using both HA as a bone growing agent and magnetically controlled Fe₃O₄ nanoparticles were explored for the first time. Fe₃O₄ nanoparticles are generally considered biocompatible and have been well investigated in non-bone related research [111]. Briefly, Fe₃O₄ nanoparticles have been used widely in vitro and in vivo as magnetic resonance imaging (MRI) contrast agents, hyperthermia agents for cancer treatment, cell separation, targeted drug delivery and tissue repair [218]. It is believed that this delivery strategy can provide a more direct and more effective way to treat osteoporosis. Most importantly, results of this study
showed that osteoblast proliferation and differentiation increased in the presence of greater concentrations (up to 200 µg/mL) of these novel HA-coated Fe₃O₄ nanoparticles which are critical events towards reversing osteoporosis [219].

2.2 Materials and methods

2.2.1 Nanoparticle synthesis

The magnetite nanoparticles used here were prepared by a wet chemistry method as previously described [220]. The chemical reaction is as follows:

$$\text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH} \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O}$$

Specifically, iron (II) chloride (Alfa aesar) and iron (III) chloride (Sigma Aldrich) at a molar ratio of 1:2 were dissolved in 100 mL of deoxygenated water at room temperature. The resulting solution was added dropwise for 5 minutes with 25 mL of a NH₄OH 28% solution (VWR) under vigorous stirring using a magnetic stir bar and nitrogen flow. A black precipitate of magnetite (Fe₃O₄) was produced. The precipitate was magnetically decanted for 10 minutes and the supernatant was removed. 100 mL of NH₄OH 5% was added to the supernatant to finish the reaction. The solution was stirred and 5g of citric acid (Sigma Aldrich) was added. Magnetite nanoparticles were heated on a hot plate at 90°C for 30 minutes to remove excess ammonium hydroxide. The solution was centrifuged at 5000 rpm for 10 minutes, washed three times with deoxygenated water and dispersed in 50 mL of deoxygenated water.

A well-established wet chemistry process was adopted to synthesize HA [221]. The chemical reaction is as follows:

$$10\text{Ca(NO}_3)_2 + 6(\text{NH}_4)_2\text{HPO}_4 + 8\text{NH}_4\text{OH} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 6\text{H}_2\text{O} + 20\text{NH}_4\text{NO}_3$$
Briefly, a 1M calcium nitrate solution (Fisher Scientific) containing iron oxide nanoparticles was added dropwise for 5 minutes into a 0.6M potassium phosphate (Sigma Aldrich) solution while vigorously stirring. The precipitation was later treated hydrothermally at 200°C for 20 hours to obtain nanocrystalline HA. Briefly, the precipitation was placed inside a PTFE container (Parr Instrument Co), which was placed in another steel container. The whole system was heated in a furnace (Lingberd/Blue). The HA precipitates were washed three times with deionized water. The precipitation was directly dried in a vacuum oven (VWR) at 50°C for 20 hours. The dried sample was ground and autoclaved using a dry cycle for 30 minutes for sterilization before use.

2.2.2 Nanoparticle characterization

To characterize the size and shape of the synthesized nanoparticles, a droplet of nanoparticles was placed on a transmission electron microscope (TEM) copper grid and allowed to dry. Imaging was carried out on a Phillips EM420 TEM.

Particle size was measured using the dynamic light scattering (DLS) technique (Malvern Zetasizer Nano). Specifically, particle solutions were diluted in water to about 200 µg/mL and 1 mL of the dilution was placed into the instrument specific container. The hydrodynamic size of particles was determined from averaging 12 different measurements and the particle size distribution was reported.

The zeta potential of the particles was measured by a Zetasizer Nano ZS90 instrument (Malvern). A similar sample preparation method was used for size and zeta potential measurements. Zeta potentials were averaged from 6 separate measurements. Water was used as the dispersant for the measurements.
The magnetic properties (via a hysteresis loop) of the dried powders were obtained using a vibrating sample magnetometer (VSM - LakeShore 7400) at room temperature. The magnetic field range was from – 1.5 T to 1.5 T. The measured magnetization was normalized to the weight of powder and reported as emu/g. The data was presented as a magnetic hysteresis loop for each material.

X-ray diffraction (XRD) was used to determine the chemical and crystalline content of the Fe$_3$O$_4$ and HA coated Fe$_3$O$_4$ powder (Siemens Diffractionmeter D5000 Kristalloflex; Bruker AXS Inc). The powder was placed on a homemade XRD glass holder. The 2θ angle was varied from 20° to 90°. Diffraction signal intensity was recorded and processed using DiffracPlus: TexEval (Bruker AXS, Inc.) software to match with HA and Fe$_3$O$_4$ standards.

2.2.3 Nanoparticle uptake by osteoblasts

Osteoblasts (human osteoblasts, population number 9 (CRL-11372, ATCC)) were used throughout the experiments. The cells were prepared from frozen stock by thawing in a 37°C water bath for one to two minutes. The cells were quickly transferred into T-flasks (Fisher) or petri dishes with Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT) and 1% penicillin/streptomycin (P/S, Hyclone, UT). Osteoblasts were grown until 90% confluent before being used in further experiments. The cells were separately incubated with Fe$_3$O$_4$ nanoparticles (200 µg/ml) and HA coated Fe$_3$O$_4$ nanoparticles (200 µg/ml) to study nanoparticle uptake. After one day, the cells were washed with phosphate buffer saline (PBS) once and added with a 1 mL Trypsin EDTA 1X solution (Sigma). Cells were further incubated for three minutes for complete detachment. The cell solution was
transferred to a 15 mL centrifuge tube (BD Falcon) and collected by centrifugation at 1200 rpm for three minutes. The supernatant was discarded and the cells were fixed with 5ml of 2.5% glutaraldehyde (Sigma) in 0.1M sodium cacodylate (Sigma) and 0.1M sucrose pH 7.4 buffer (Sigma) for one hour. The cell pellet was then washed in 0.1M sodium cacodylate buffer at pH 7.4 and 0.1M sucrose three times by centrifugation. To enhance contrast of the images, 1% osmium tetroxide (Electron Microscopy Science) was added to the solution. After samples were washed three times with water, the cells were centrifuged vigorously (7000 rpm, 15 minutes) to form a firm pellet. The cell pellet was dehydrated using a series of ethanol solutions (Sigma) at 25%, 50%, 75%, 95% and 100% (20 to 40 minutes each time). Cells were then transferred into epoxy resins (Electron Microscopy Science). Resin – ethanol ratios were increased gradually from 1:2 (three hours), 1:1 (overnight), 3:1 (three hours) to 1:0 (overnight). The final resins were cured in an oven at 60°C for 18 hours. Samples were cut into 90nm thick slides by a microtome FSC-902100A (Thermo Fisher) and placed on copper grids. Imaging was carried on a Phillips EM 410 TEM at 100 kV.

2.2.4 Osteoblast proliferation

Osteoblast (human osteoblasts population number 9 (CRL-11372, ATCC)) proliferation tests were conducted at one, three, and five days. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT) and 1% penicillin/streptomycin (P/S, Hyclone, UT) in a 96 well plate at a volume of 200 µL per well and a seeding density of 3500 cells/cm² was used. Cells were seeded in the presence of 100 µg/mL Fe₃O₄ nanoparticles, 100 µg/mL HA coated Fe₃O₄ nanoparticles, and a control with no nanoparticles. Background
solutions containing no cells were also prepared using DMEM supplemented with the same concentrations of nanoparticles (100 µg/mL) as those exposed to cells. Each experiment was replicated three times. Nanoparticles were sonicated at 90W (Ultrasonic Cleaner 75D, VWR) for 15 minutes and vortexed (Fisher Scientific Vortex Mixer) before being added to the cells. Cells were incubated under standard conditions (37°C, humidified, 5% CO₂, 95% air environment) for one, three and five days. At the end of each incubation period, an MTS assay was performed using the CellTiter96 assay (Promega). The CellTiter96 assay is a colorimetric method to determine cell viability after proliferation. For this, after each time period, 40 µL of CellTiter96 was added to each well of the 96-well plate. The cells were incubated for three more hours and then analyzed using a microplate reader (SpectraMax300, Molecular Devices) at 490 nm for absorbance. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. To correlate optical density with cell number, a standard curve was established. For the standard curve, cells were seeded as high as 140,000 cells/cm² in a 96-well plate. The cells were serial diluted by a factor of 2 10 times and the MTS assay was performed immediately. A linear correlation was obtained between known cell numbers and optical density.

2.2.5 Osteoblast differentiation assay

Osteoblast intracellular protein synthesis, alkaline phosphatase (ALP) activity, collagen synthesis and extracellular calcium deposition (important osteoblast differentiation markers) were determined in the present study in the presence of HA coated Fe₃O₄ nanoparticles.
For this, osteoblasts (human osteoblasts, population number 9 (CRL-11372, ATCC)) were seeded on polystyrene cell culture plates (BD Falcon) at a density of 100,000 cells/cm² and cultured in DMEM supplemented with 10% FBS, 1% P/S, 50 mg/mL L-ascorbate (Sigma Aldrich) and 10 mM β-glycerophosphate (Sigma Aldrich) under standard cell culture conditions for 7, 14, and 21 days. HA coated Fe₃O₄ nanoparticles were added to cells at concentrations of 200 µg/mL, 100 µg/mL, and 12.5 µg/mL. Medium was replaced every other day with fresh media not containing nanoparticles. At the end of each prescribed time period, the supernatant medium was removed and the substrates were rinsed with tris buffered saline (TBS - pH 7.2, Sigma Aldrich) for three times. The remaining osteoblasts on the substrates were lysed using 500 µL of deionized water and three freeze-thaw cycles. Briefly, the substrates were frozen in a -80°C freezer for three hours and thawed at room temperature for three hours. The cycle was repeated three times. The supernatant lysates were transferred into microtubes (Eppendorf micro-centrifuge tube) for determining intracellular protein synthesis, ALP activity and collagen synthesis, while the remaining substrates were used for determining extracellular calcium deposition.

For each prescribed time period, experiments were performed in triplicate and repeated three times.

2.2.5.1 Total intracellular protein synthesis

The total intracellular protein content in the cell lysates was determined using a commercially available kit (Pierce Chemical) via a spectrophotometer (SpectraMax300, Molecular Devices) and following the manufacturer’s instructions. For this purpose, aliquots of each protein-containing, distilled-water supernatant were incubated with a
solution of copper sulfate and bicinchoninic acid (BCA) at 37°C for two hours. Cu$^{2+}$ was reduced to Cu$^{1+}$ by protein in an alkaline medium. The BCA then reacted with a reduced cation (Cu$^{1+}$) to develop an intense purple-colored product. Light absorbance of these samples was measured at 562 nm. Total intracellular protein synthesized by osteoblasts was determined from a standard curve of absorbance versus known concentrations of albumin (bovine serum albumin, Sigma) run in parallel with experimental samples. Substrates with osteoblasts but no nanoparticles served as controls.

2.2.5.2 **Intracellular Alkaline Phosphatase (ALP) activity**

Intracellular ALP activity is an indicator of how active osteoblasts are in producing new bone. Intracellular osteoblast ALP activity was analyzed by a commercial ALP activity detection kit (Upstate Cell Signal Solutions) following manufacturer’s instructions. The ALP activity in cell lysates was detected using a spectrophotometer (SpectraMax300, Molecular Devices) by adding a Malachite Green solution (Upstate Cell Signal Solutions) compared to a standard curve of absorbance versus concentrations of converted phosphate, which reported ALP activity as the amount of converted phosphate. ALP activity was further normalized by total intracellular protein content and substrate surface area.

2.2.5.3 **Intracellular collagen synthesis**

Sirius red is a strong anionic dye, which has been used for the quantification of collagen for many years. Sulphonic acid groups in Sirius red react with basic groups present in the collagen molecule and the dye molecules attached to the collagen fiber. To quantify intracellular collagen synthesis, the cell lysates were dried onto a microplate at
37°C and stained with 1% Sirius Red stain (Sigma) at room temperature for one hour. The excessive stain in the microplate was then removed by rinsing the plate five times with 0.01 M HCl (Fisher). The stained collagen was then dissolved with 0.1 M NaOH (Fisher) for one hour and the solution was replaced into another microplate for spectrophotometric analysis. Light absorbance of the solution at 540 nm was read and compared to a standard curve of absorbance versus known concentrations of collagen in solution (Collagen Type I, Sigma Aldrich). Intracellular collagen content was normalized by total intracellular protein.

2.2.5.4 Calcium deposition in the extracellular matrix

Osteoblast lysates were removed from the substrates after 21 days of incubation and the remaining extracellular matrix on the substrates was treated with 0.6 M HCl at room temperature for 24 h. After the prescribed time period, a 100 µL sample solution was dissolved in 500 µL aqua regia (a solution of HNO₃ and HCl combined at a volume ratio 1:3). Then, the aqua regia was heated at 300°C and evaporated using a hot plate. Resulting salts were dissolved in a HNO₃ solution (Sigma) at 2% for elemental analysis using the inductively coupled plasma mass spectrometry (ICP-MS) technique (JY2000 Ultrace ICP Atomic Emission Spectrometer - Horiba). The samples were first evaporated and ionized. The ions were separated according to their mass-to-charge ratio by electromagnetic fields. The ions were then detected and processed into mass spectra.

To differentiate between the added calcium in HA (in the coatings) and calcium deposited by osteoblasts, the amount of calcium from the HA coatings was calculated from the Ca/Fe ratio for the nanoparticles as described above. This amount of Ca was
subtracted from the total Ca measured to get the Ca deposited by osteoblasts. The calcium deposited by osteoblasts was normalized by substrate area.

2.2.6 Long-term osteoblast morphology under scanning electron microscopy (SEM)

To visualize osteoblast morphology in the presence and absence of the nanoparticles, osteoblasts were seeded on glass substrates (circular glass slides, VWR) similar to those used in the long-term osteoblast assays. These glass substrates were cleaned by ethanol and acetone soakings and treated with NaOH 1M for 20 minutes. After 21 days of culture, the substrates were removed from the media using a tweezer, transferred to a new plate, rinsed with a 0.1M sodium cacodylate and 0.1M sucrose pH 7.4 buffer, and fixed with 2.5% glutaraldehyde in a 0.1M sodium cacodylate and 0.1M sucrose pH 7.4 buffer for 30 minutes. The samples were then dehydrated with 30%, 50%, 70%, 80%, and 95% ethanol for 10 minutes each and 100% ethanol for 10 minutes (3 times) and finally 100% ethanol for 40 minutes. The samples were then critically-point dried in CO$_2$ and sputter coated with Au/Pt before examination with SEM (LEO 1530VP, Zeiss, field emission gun operating at 5 kV).

2.2.7 Statistical analyses

Results were analyzed using Student T-tests and data were expressed as the mean ± standard error of the mean (SEM).
2.3 Results

2.3.1 Nanoparticle synthesis and characterization

TEM images (Figure 2.1a) demonstrated that Fe$_3$O$_4$ with diameters ranging from 5 to 20 nm were successfully synthesized in this study. HA had rod shapes similar to those obtained in previous research [222]. Nano HA had average sizes 60 nm long and 20 nm wide (Figure 2.1). TEM images also showed that iron oxide nanoparticles were embedded in the HA coatings.

![Figure 2.1 TEM micrographs of the synthesized (a) Fe$_3$O$_4$ and (b) HA coated Fe$_3$O$_4$ nanoparticles. The arrow indicates a Fe$_3$O$_4$ nanoparticle coated with a HA nanoparticle.](image)

Size distributions of Fe$_3$O$_4$ and HA coated Fe$_3$O$_4$ nanoparticles were provided by DLS (Figure 2.2), where the average hydrodynamic size of Fe$_3$O$_4$ nanoparticle was 20 nm and for HA coated Fe$_3$O$_4$ was 170 nm. The distributions show that Fe$_3$O$_4$ nanoparticles were mostly monodispersed while agglomeration of HA coated Fe$_3$O$_4$ nanoparticles contained 2 to 3 individual nanoparticles.
Figure 2.2 Size distribution of Fe$_3$O$_4$ nanoparticles (upper graph) and HA coated Fe$_3$O$_4$ nanoparticles (lower graph) obtained by dynamic light scattering (DLS).

The nanoparticle powder was confirmed to possess crystalline Fe$_3$O$_4$ (Figure 2.3) and HA and Fe$_3$O$_4$ by XRD (Figure 2.4) where the intensity peaks in XRD patterns matched well with HA and Fe$_3$O$_4$ (magnetite) standards. XRD patterns of Fe$_3$O$_4$ nanoparticles showed characteristic peaks of magnetite at 2$\theta$ equals 30.17°, 35.46°, 43.38°, 57.23°, and 62.77°. The peaks in XRD patterns for HA coated Fe$_3$O$_4$ nanoparticles mostly belonged to HA. The intensity of Fe$_3$O$_4$ peaks were small compared to those of HA since the Fe/Ca ratio was low. Using the Scherrer equation, the size of Fe$_3$O$_4$ and HA coated Fe$_3$O$_4$ nanoparticles were calculated to be 13 and 50 nm, respectively. The calculation did not count on the size effect of HA nanoparticles, but the
sizes obtained were very close to what were observed in TEM images and measured in DLS experiments.

Figure 2.3 X-Ray diffraction (XRD) patterns of Fe$_3$O$_4$ nanoparticles.

Figure 2.4 X-Ray diffraction (XRD) patterns for the HA coated Fe$_3$O$_4$ nanoparticles.
According to the vibrating sample magnetometry (VSM) results, the synthesized Fe$_3$O$_4$ nanoparticles were superparamagnetic, meaning that the nanoparticles will not retain magnetic properties after removal of a magnetic field (Figure 2.5a). The nanoparticles showed high saturation magnetization values of about 50 emu/g. After being coated with HA, the nanoparticles still showed similar superparamagnetic properties (Figure 2.5b). However, due to high Ca/Fe ratios, the saturation magnetization per gram of material reduced. By increasing the amount of Fe$_3$O$_4$, higher magnetization can be obtained, thus, potentially being more suitable for magnetically controlling their distribution to bone defect areas using an external magnetic field.

![Magnetization curves of (a) Fe$_3$O$_4$ and (b) HA coated Fe$_3$O$_4$ nanoparticles obtained by a vibrating sample magnetometer (VSM).](image)

Zeta potential measurements of Fe$_3$O$_4$ nanoparticles and HA coated Fe$_3$O$_4$ nanoparticles in water gave values of -40 and -60 mV, respectively. These values suggested that the nanoparticles were stable in water which was due to the electrostatic repulsion between the charged particles. A stable nanoparticle is more desirable for drug delivery applications, since the nanoparticles can stay longer in circulation.
2.3.2 Nanoparticle uptake by osteoblasts

After one day of incubation, Fe$_3$O$_4$ nanoparticles were observed inside osteoblasts (Figure 2.6). The nanoparticles were found mostly in the vesicles of osteoblasts. Nanoparticle sizes in the cells were less than 20 nm which might indicate a size exclusion of the nanoparticles that can penetrate into osteoblasts. There were particles outside of the cells, but they tended to aggregate into larger clusters (>500 nm). The particles might have been coated with serum proteins, which accelerated the formation of agglomerates.

![Figure 2.6 TEM micrographs of Fe$_3$O$_4$ uptake by osteoblasts after one day of incubation. Arrows demonstrate nanoparticle positions. Scale bar = 100 nm.](image)

On the contrary, many HA coated Fe$_3$O$_4$ nanoparticles were found outside the cells and attached to the cell membranes (Figure 2.7a). This was expected since the size of the coated nanoparticles (~ 60 nm long) was larger than that of the iron oxide nanoparticles alone (less than 20 nm). Large aggregates of HA coated nanoparticles, which could be taken into the cells via phagocytosis or receptor-mediated endocytosis, were also found in the cells (Figure 2.7b).
Figure 2.7 TEM micrographs of HA coated Fe$_3$O$_4$ uptake by osteoblasts after one day of incubation. The nanoparticles were found attached to the cell surfaces (a) and inside cell (b). Scale bar = 100 nm.

2.3.3 Osteoblast proliferation

Osteoblast proliferation tests conducted at one, three and five days showed that HA coated Fe$_3$O$_4$ increased osteoblast density compared to the controls (no particles) (Figure 2.8). After five days of culture, osteoblasts exposed to non-coated Fe$_3$O$_4$ showed some toxicity. The cell density (76,000 cells/cm$^2$) was lower than the control (98,000 cells/cm$^2$). After one day and three days, osteoblast densities were similar in the presence of HA coated magnetic nanoparticles compared to the control. However, after five days, samples with HA coated Fe$_3$O$_4$ had significantly higher osteoblast densities (108,000 cells/cm$^2$) compared to the control (p<0.05). This result demonstrated that with an HA coating, the magnetic nanoparticles significantly enhanced osteoblast proliferation. As described next, the long-term cell function experiments gave additional evidence of the ability of using these nanoparticles to treat osteoporosis.
Figure 2.8 Increased osteoblast density in the presence of HA coated Fe$_3$O$_4$ after 1, 3 and 5 days of culture. Nanoparticle concentration was 100 µg/mL. Data = mean ± Standard Error of the Mean (SEM); N = 3. *p< 0.01 compared to the same sample at earlier time points, **p< 0.05 compared to the Fe$_3$O$_4$ sample at the same time points, ***p< 0.05 compared to the control sample at the same time points.

2.3.4 Osteoblast differentiation assays

Results of ALP activity using 200 µg/mL HA coated magnetic nanoparticles showed significantly higher ALP activity compared to control samples at all time periods (Figure 2.9). After 21 days, osteoblasts from the 200 µg/mL HA coated magnetic nanoparticle concentration had significantly enhanced ALP activity compared to all other three concentrations. For the 200 µg/mL and 100 µg/mL concentrations, ALP activity did not change significantly over time. However, there was an increasing trend of ALP activity over time for 12.5 µg/mL concentrations and the control. ALP activity is a marker of bone formation and plays a key role in bone mineralization by initiating and
promoting the formation of HA in matrix vesicles of osteoblasts then propagating them into the extracellular matrix [18].

Figure 2.9 Alkaline phosphatase (ALP) activity of osteoblasts after 7, 14, and 21 days of culture in the presence of HA coated nanoparticles at concentrations of 12.5, 100, and 200 µg/mL. Data = mean ± SEM; N = 3. ** p<0.05 compared to same sample at the earlier time periods, *p<0.05 compared to other samples at the same time points.

After 21 days, osteoblasts cultured in the presence of the 200µg/mL HA coated iron oxide nanoparticles had the highest levels of collagen, which was significantly higher than controls (Figure 2.10). Collagen type I is the main organic component of natural bone. The fact that osteoblasts cultured in the presence of the 200µg/mL HA coated iron oxide nanoparticles deposited the highest amount of collagen provided evidence of the formation of new bone.
Figure 2.10 Collagen synthesis by osteoblasts after 21 days of culture in the presence of HA coated nanoparticles at concentrations of 12.5, 100, and 200 µg/mL. Data = mean ± SEM; N=3. *p<0.1 compared to control samples.

One of the most important markers regarding bone formation and osteoblast differentiation is clearly calcium deposition in the extracellular matrix. Osteoblasts cultured with HA coated nanoparticles (all concentrations) deposited more calcium compared to controls (Figure 2.11). Especially, calcium deposited increased by 10 times by osteoblasts cultured in the presence of the 200 µg/mL HA coated iron oxide nanoparticles compared to controls. This result was also consistent with the results of ALP activity and further confirmed better osteoblast differentiation in the presence of HA coated Fe₃O₄ nanoparticles than controls (no particles).
Figure 2.11 Extracellular calcium deposition by osteoblast in the presence of HA coated Fe$_3$O$_4$ nanoparticles after 21 days of culture. Data = mean ± SEM. *p<0.01 compared to control samples. **p < 0.05 compared to samples with smaller amounts of nanoparticles.

2.3.5 Long-term osteoblast morphology under scanning electron microscopy (SEM)

Figure 2.12 and Figure 2.13 show osteoblasts after 21 days of culture in the presence of HA coated Fe$_3$O$_4$ (200 µg/mL sample) and without nanoparticles (control sample). In Figure 2.12, many nanoparticles are visible on the osteoblasts. Using EDS, these particles were confirmed to be HA (inset of Figure 2.12). The morphology of osteoblasts was also different between the two samples. While the osteoblast filopodia network was extensive in the presence of the HA coated iron oxide nanoparticles, no such osteoblast filopodia network was apparent for the control sample (Figure 2.13).
Figure 2.12 SEM images of osteoblasts after 21 days of culture in the presence of 200 µg/mL HA coated Fe$_3$O$_4$ nanoparticles. The smaller image shows a magnified cell with nanoparticles attached on surface. The particles were confirmed as HA by energy-dispersive X-ray (EDS) spectroscopy. Scale bar = 20 µm. Inset image width = 20 µm.
Figure 2.13 SEM images of osteoblasts after 21 days of culture without nanoparticles. The smaller image shows a magnified cell with no particles and less filopodia. Scale bar = 20 µm. Inset image width = 21 µm.
2.4 Discussion

2.4.1 Material synthesis

In this study, in order to create a new approach for treating bone defects (such as osteoporosis), nanoparticles were created that could potentially be directed to a desired bone site to induce bone formation using an external magnetic field. HA coated Fe₃O₄ nanoparticles were that material created here. The nanoparticles created were the combination of magnetically controllable Fe₃O₄ and the osteoblast “preferable” HA chemistry. Separately, Fe₃O₄ and HA are biocompatible [79] and when combined, the nanoparticles enhanced several important osteoblast markers of differentiation, such as ALP activity, collagen synthesis and most importantly calcium deposition, while still retaining magnetic properties for directional control in the body.

2.4.2 A proposed mechanism of osteoblast proliferation and functional enhancement related to protein adsorption on the nanoparticle surface

It is recognized that surface chemistry, surface energy and surface topography have large influences on osteoblast responses [35, 223-228]. It has been shown that among those properties, surface topography contributes greatly. Previous studies have demonstrated that by adding nanometer surface features to traditional implant surfaces, enhanced osteoblast adhesion, proliferation and differentiation can be obtained [18]. For example, Webster et al. tested osteoblast proliferation on three different surfaces of different chemistry but similar nanometer topographies [215]. The results showed that bone cell proliferation was enhanced on all nanophase ceramic surfaces compared to micron structured ceramic surfaces regardless of chemistry.
In the current study, the addition of HA coated iron oxide nanoparticles increased both osteoblast proliferation and differentiation. The reasons behind such improvements are still largely under investigation. However, a common hypothesis is protein adsorption and bioactivity on surfaces or particles with nanometer features is different from that on conventional nanometer smooth surfaces (Figure 2.14) [44, 215, 229]. A cell interacts with a biomaterial via receptors on the cell surface and a layer of proteins adsorbed onto material surfaces. The receptors can only recognize certain type of proteins. In this case, specific proteins (such as fibronectin and vitronectin) adsorb to nanoparticle surfaces and enhance osteoblast spreading and proliferation. Previously, Webster et al. showed that of all proteins contained in serum, vitronectin adsorbed in the highest concentration to nanophase surfaces of alumina while albumin adsorbed in the greatest amount on conventional alumina [215]. Vitronectin is known to be crucial for osteoblast adhesion since surfaces treated with vitronectin greatly enhance osteoblast adhesion compared to other proteins contained in serum [44]. In this study, vitronectin or fibronectin may have adsorbed in greater amounts to the HA coated iron oxide nanoparticles. The subsequent interaction between the HA coated iron oxide nanoparticles and osteoblast membranes may have enhanced osteoblast proliferation and differentiation by triggering key integrin receptors (such as α5β1, αIIbβ3 and αVβ3 integrins on cell membranes). It is also possible that the HA nanoparticles uptaken by osteoblasts were dissolved in lysosomes. Consequently, the increased Ca^{2+} inside cells would enhance osteoblast functions.
Figure 2.14 The interaction of a cell and a biomaterial is influenced by the adsorption and bioactivity of proteins to biomaterial surfaces. The cell is demonstrated as a large circle with many adhesion receptors protruding. Proteins in media are shown as squares, circles and triangles. The cell membrane receptors can only recognize certain types of proteins (in this case the solid circle). Consequently, the cell can only adhere to certain surface-bound proteins [230].
2.4.3 **Cell morphology changed by nanotopology**

Moreover, the results from this study also showed changes in cell morphology in the presence of HA coated magnetic nanoparticles indicating improved osteoblast differentiation. Specifically, after 21 days, for the samples with HA coated magnetic nanoparticles, there was an extensive network of osteoblast filopodia, protruding in all directions. In comparison, the control sample showed less osteoblast filopodia and less protrusion. The importance of filopodia extension on surfaces with nanometer features (and its relationship to focal point adhesion and consequently cell adhesion) was noted by Yang *et al.* [231]. They found that cell filopodia were longer and protruded in all directions more on nanofeatured diamond surfaces, which led to increased osteoblast differentiation on nanofeatured compared to nanosmooth diamond [231].

Moreover, Arnold *et al.* demonstrated that adhesive spots separated by more than 78 nm limited osteoblast attachment and spreading [232]. Although further investigation is needed, it is plausible that by adding HA coated magnetic nanoparticles (largest dimension < 70 nm) to osteoblasts, the number of cell adhesion sites increased (especially if coated with fibronectin), leading to enhanced osteoblast proliferation and differentiation. On the other hand, the non-HA-coated magnetic nanoparticles might have attracted other non-specific proteins for osteoblast such as albumin or laminin, which led to lower cell density compared to the control samples.

2.4.4 **Challenges in the clinical applications of HA coated magnetic nanoparticles**

It is very important to note that one of the major challenges for magnetic therapeutic systems, however, lies in the ability to control those nanoparticles once
injected in the body. Larger particles have a higher saturation magnetization and are, thus, easier to control in the body. However, particle sizes have an upper limit of about 100 nm in order to successfully invade the reticuloendothelial system [111]. An individual HA coated nanoparticle created in this study is smaller than that stated limit. However, particle agglomeration (especially in cell media supplemented with serum) can easily increase the particle size; therefore further studies should be done on dispersing these nanoparticles. Regarding magnetic control, many efforts have been made, yet the control system is still very simple consisting of a permanent magnet placed close to the desired location of magnetic nanoparticle assembly [127, 137, 185-186]. This strategy lacks accuracy as the magnetic field can only penetrate as deep as 8 – 12 cm in the body [187-188]. Recent studies have developed an algorithm to control magnetic nanoparticle location dynamically [190-192, 233]. These results seem to be very promising; however, controlling nanoparticles in vivo is significantly more complicated than in simulation and in vitro settings. A more thorough calculation regarding magnetic forces acting on a nanoparticle will be presented in chapter 6.

2.5 Conclusions

Fe$_3$O$_4$ and HA coated Fe$_3$O$_4$ nanoparticles were synthesized and characterized using TEM, DLS, XRD and VSM in this part of the study. Osteoblast proliferation and differentiation were studied in the presence of uncoated and HA coated Fe$_3$O$_4$ nanoparticles. The results showed that HA coated Fe$_3$O$_4$ nanoparticles were magnetic and enhanced osteoblast density after 5 days of culture. Most importantly, using these nanoparticles at concentrations of 200 µg/ml improved osteoblast differentiation after 21 days of culture, as shown by greater ALP activity, collagen synthesis and calcium
deposition compared to control samples without any nanoparticles. These results highlight the potential of using HA coated magnetic nanoparticles to increased bone growth at desirable bone defects sites which should be further studied.

To further understand the mechanism of how the nanoparticles interact with bone cells to enhance bone cell function, it is very important to investigate protein adsorption and bioactivity on nanoparticle surfaces, the uptake of nanoparticles into cells and the regulation of genes influenced by the presence of nanoparticles. These issues will be addressed in the next chapter.
CHAPTER 3. PROTEIN ADSORPTION ON NANOPARTICLES AND
OSTEOBLAST GENE REGULATION IN THE PRESENCE OF HA COATED
Fe$_3$O$_4$ NANOPARTICLES

3.1 Introduction

In the previous chapter, Fe$_3$O$_4$ and HA coated Fe$_3$O$_4$ nanoparticles were synthesized and characterized. The HA coated nanoparticles showed not only short term enhancement in osteoblast proliferation but also long term improvement in osteoblast differentiation markers (such as alkaline phosphatase activity, collagen synthesis and calcium deposition) compared to the control samples (without nanoparticles). These nanoparticles are, thus, very promising for osteoporosis treatments. In this chapter, the thesis will address the mechanisms of the improved osteoblast response in the presence of HA coated Fe$_3$O$_4$ nanoparticles. First, evidence that will be presented which shows altered protein adsorption and bioactivity (such as fibronectin) on nanoparticle surfaces. Lastly, osteoblast gene regulation (osteocalcin, type I collagen and cbfa-1) results will be presented in the presence of nanoparticles. Collectively, these results provide a mechanism for the enhanced osteoblast functions in the presence of HA coated iron oxide nanoparticles.

3.1.1 Protein adsorption onto biomaterials surfaces

It is widely accepted that the interaction between a cell and a biomaterial occurs via proteins adsorbed onto the biomaterial surface [234]. Whenever a biomaterial is placed into the body (such as a prosthesis that is implanted or a drug that is injected into the body), it is immediately (within seconds) coated with proteins. Consequently, cells
respond to biomaterials through a layer of proteins adsorbed onto a material surface. These proteins (or smaller region of proteins) act as ligands to communicate with specific receptors on the cell surface. Since cell membrane receptors can only recognize specific ligands, biomaterials that adsorb different proteins on the surface promote the adhesion of different types of cells. For example, the arginine-glycine-aspartic acid (RGD) sequence has been shown to facilitate cell integrin and biomaterial interactions [235-237]. This interaction is very important because it regulates cell migration, growth, differentiation and apoptosis. Several extracellular matrix proteins (such as collagen, fibronectin and vitronectin) containing RGD are known to regulate osteoblast spreading and adhesion [237-238]. For instance, fibronectin communicates with osteoblast specifically via $\alpha_5\beta_1$ integrin receptors. By blocking these receptors, binding of osteoblasts to Tivanium (Ti6A14V) and Zimaloy (CoCrMo) decreased 63% and 49%, respectively [239]. A major protein in plasma, albumin, is considered to be a non-specific protein for controlling osteoblast interactions [240-241].

3.1.2 Gene regulation influenced by biomaterials

To understand the molecular mechanisms of osteoblasts and HA coated $\text{Fe}_3\text{O}_4$ nanoparticle interactions, it is also important to elucidate the gene-expression profile of osteoblasts in the presence of such nanoparticles. For bone cells, it is very important to examine the expression of type I collagen, osteocalcin and cbfa-1 genes. Type I collagen, which is secreted in large quantities by osteoblasts, is the most abundant collagen in vertebrates [242]. It contributes to 90% of the organic phase of bone mass. Osteocalcin is one of the most abundant non-collagenous proteins in bone [243]. Osteocalcin is expressed at high levels only in differentiated osteoblasts and odontoblasts (the
counterparts of osteoblasts in teeth) [244]. Since it is very specific for osteoblasts, osteocalcin is often used as a bone formation marker. Cbfa-1 is one of the transcriptional factors that regulates osteoblastic differentiation and bone formation [245]. Cbfa-1 also correlates to the regulation of other genes including alkaline phosphatase, collagen, and osteopontin [246]. Therefore, in this part of the thesis, the expression of three genes, osteocalcin, type I collagen and cbfa-1, was determined by the quantitative real-time polymerase chain reaction method (RT-PCR). The results will elucidate the influence of HA coated Fe$_3$O$_4$ nanoparticles on osteoblast gene expression, which can be linked to the aforementioned protein adsorption and greater osteoblast functions.

3.2 Materials and methods

3.2.1 Fourier transform infrared (FTIR) spectra of nanoparticles

FTIR is a technique that identifies the type of chemical bonds in molecules by obtaining an infrared absorption spectrum [247]. When a material is exposed to infrared radiation, the molecules in that material absorb only certain frequencies, called resonant frequencies, which characterize their structure (i.e. the chemical bonds or groups that vibrate). The frequencies are matched with the chemical bonds that vibrate and, thus, the types of bonds can be determined.

In FTIR spectroscopy, the infrared light containing many different frequencies is emitted from the source, guided through a collimator and to a Michelson interferometer (Figure 3.1). The interferometer consists of a beam splitter and two mirrors, one fixed and one moveable. Light reflects back from the two mirrors to the beam splitter and is focused on the sample compartment. The light leaving the sample is refocused on a
detector. By changing the position of the moving mirror, the distribution of infrared radiation is altered resulting in an interferogram. The raw signal is represented as intensity with respect to mirror position. Using Fourier transformation, the raw data can be converted into light output as a function of wavelength or wavenumber.

Figure 3.1 A simplified diagram of a Fourier transform infrared (FTIR) interferometer.

To determine the FTIR spectra of nanoparticles, solutions of 200 µg/mL of HA coated Fe₃O₄ (prepared in section 2.2.1 of Chapter 2) in DMEM supplemented with 10% fetal bovine serum (FBS) were prepared. Glass slides (VWR, USA) were placed in each well of the 12-well tissue culture plate (Corning). 1 mL of each solution was place in each well and allowed to dry for 48 hours. For comparison purposes, the same sample was dried without serum and the powders were used for FTIR measurements. The measurements were conducted in the wavelength range from 2-25 µm at a wavenumber resolution 4 cm⁻¹. The measurements were carried on a Thermo Nexus 870 FTIR
spectrometer. Reflectance with respect to wavenumber was reported. The dips in the FTIR spectra were then compared to standards to determine the type of chemical bonds and possible protein adsorption indicated.

3.2.2 Albumin adsorption

The amount of albumin adsorbed on nanoparticle surfaces was determined using a commercially available bicinchoninic acid assay (BCA) total protein kit (Thermo Scientific) via a spectrophotometer (SpectraMax300, Molecular Devices) and following the manufacturer’s instructions. For this purpose, solutions of HA coated Fe$_3$O$_4$ nanoparticles (prepared as described in section 2.2.1 of Chapter 2) with concentrations at 12.5, 100 and 200 µg/mL were prepared in micro centrifuge tubes (Fisher). Each tube was supplemented with bovine serum albumin (BSA - Sigma) to a 600 µg/mL solution. This amount of albumin was comparable to the amount albumin in DMEM solutions (~2000 µg/mL) [248] and in the range of detection of the BCA kit (20 – 2000 µg/mL). The tubes were incubated at 37°C and shaken at 200 rpm for two hours. At the end of the incubation period, the nanoparticles were collected by centrifugation at 10,000 rpm for 10 minutes. 200 µL of the supernatants were incubated with a solution of copper sulfate and bicinchoninic acid at 37°C for two hours. Light absorbance of these samples was measured at 562 nm. The amount of albumin in the supernatants was determined from a standard curve of absorbance versus known concentrations of albumin (bovine serum albumin, Sigma) run in parallel with experimental samples. The amount of adsorbed albumin was determined by subtracting the amount of albumin added to each solution from the amount of albumin in the supernatant.
3.2.3  Fibronectin enzyme-linked immunosorbent assay (ELISA)

Fibronectin is a large adhesive glycoprotein (MW \( \sim 450,000 \text{ Da} \)) (Figure 3.2). It is heterodimeric and consists of 31 globular repeating units with multiple surface receptors and binding sites for matrix components [249]. Fibronectin plays an important role in material/cell interactions because of its ability to attach osteoblasts to the extracellular matrix. The RGD sequence is located at FN III\(_{10}\) and is the primary site for cell attachment via \( \alpha 5\beta 1 \), \( \alpha IIb\beta 3 \) and \( \alpha V\beta 3 \) integrins on cell membrane [250]. Therefore, the adsorption of fibronectin on biomaterial surfaces is crucial for cell adhesion, migration and spreading.

![Figure 3.2 Domain organization of fibronectin [251]. The model shows the distribution of disulfide bonds and the binding sites for relevant proteins and monoclonal antibodies 4D1, 7D5 and 5C3.](image)

Fibronectin adsorption onto nanoparticle surfaces was measured by an enzyme-linked immunosorbent assay (ELISA). ELISA is a biochemical technique that is widely used in immunology to detect the presence of an antigen and antibody in samples. In this specific case, fibronectin was linked with an anti-fibronectin molecule. This antibody was
then detected by a secondary antibody, which was functionalized with a fluorescence molecule. From the fluorescence signal read by a spectrophotometer, the amount of fibronectin was determined.

Specifically, HA coated Fe$_3$O$_4$ solutions at concentrations of 12.5, 100 and 200 µg/mL were prepared in DMEM supplemented with 10% FBS and 1% P/S. Nanoparticle solutions at the same concentrations were also incubated in DMEM without serum and were treated as controls.

The solutions were shaken at 200 rpm at 37°C for 20 minutes before the nanoparticles were collected via centrifugation at 10,000 rpm for five minutes. Samples were dispersed in PBS and centrifuged at 10,000 rpm for 5 minutes twice to remove proteins that did not adsorb to the nanoparticles. 500 µL of 2% BSA (wt% in PBS) was added to the nanoparticle solution to block all unbound sites. After one hour, the nanoparticles were collected by centrifugation at 10,000 rpm for five minutes and rinsed twice with PBS. A rabbit anti-bovine fibronectin antibody (AB2047, Chemicon) was added to the nanoparticles at 3 µg per 500 µL of a 1% BSA solution. The solutions were mixed and incubated at 37°C for one hour for fibronectin and anti-fibronectin binding.

Samples were centrifuged at 10,000 rpm for five minutes and rinsed with a 0.05% Tween 20 (Sigma) solution three times. A secondary antibody, goat anti-rabbit conjugated with horseradish peroxidase (HRP), was added to nanoparticles at a concentration of 10 µg/mL (in 1% BSA) and incubated at 37°C, 200 rpm for one hour. Nanoparticles were collected by centrifugation at 10,000 rpm for five minutes and rinsed with 0.05% Tween 20 two times.
The nanoparticles were transferred into new microcentrifuge tubes before being supplemented with 500 µL of a 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (SK-4500, Vector Laboratories) solution to each tube. ABTS reacts only with HRP to develop a water soluble, green colored product. The solutions were covered from light and incubated at room temperature for 20 minutes.

Nanoparticles were collected by centrifugation at 10,000 rpm for five minutes. 200 µL of the ABTS reacted solutions were transferred to wells of a 96-well plate for spectrophotometer measurements. Different intensities of fluorescence were detected by a spectrophotometer (SpectraMax300, Molecular Devices) at 405 nm. Fluorescence intensities were subtracted from those of the corresponding control.

3.2.4 Cell preparation for gene regulation experiments

Osteoblasts (CRL-11372, ATCC, population number 9) were seeded on polystyrene substrates at a density of 200,000 cells/well in 6-well plates and cultured in DMEM supplemented with 10% FBS, 1% P/S, 50 mg/mL L-ascorbate (Sigma Aldrich) and 10 mM β-glycerophosphate (Sigma Aldrich) under standard cell culture conditions for 24 hours. At this point, HA coated Fe₃O₄ nanoparticles were added to cells at concentrations of 200 µg/mL, 100 µg/mL, and 12.5 µg/mL and the cells were grown for an additional 24 hours.

3.2.5 Total RNA extraction

At the end of the incubation period, 1mL of TRIzol (Applied Biosystems) was added to each well plate. The plates were incubated at room temperature for 10 minutes before cells were scrapped for total RNA isolation. The cells were collected in micro
centrifuge tubes (Eppendorf). The cell solution was added with 500 µL of chloroform (Fisher), vortex mixed and centrifuged at 13,000 rpm for 15 minutes to isolate RNA. After centrifugation, two-phase solutions appeared in each tube. The clear solution on the top containing isolated RNA (around 500 µL) was carefully transferred into another micro centrifuge tube. After the isolation, 0.5 mL of isopropyl alcohol (Fisher) was added for the RNA precipitation. The tubes were vortex mixed and incubated at room temperature for 10 minutes. Subsequently, the solution was centrifuged at 13,000 rpm for 10 minutes and the resulting pellet was washed with 750 µL of 75% ethanol twice before air drying and was dissolved in 12 µL nuclease free water (Applied Biosystems). At this point, the RNA was ready for further quantification.

RNA purity and concentration were measured by placing 2 µL of the RNA solution in a Nanodrop ND-1000 (NanoDrop) instrument. The Nanodrop instrument is a spectrophotometer that is able to measure absorbance of a solution at different wavelengths. For RNA, absorbance at 260nm and 280nm were measured against nuclease free water. The A260/A280 ratio of sample absorbance at 260 and 280 nm was used to assess purity of the isolated RNA. RNA with a 260/280 ratio of 1.8 or larger is generally considered “pure”. All samples that were used in the next steps had a 260/280 ratio larger than 1.8. The Nanodrop instrument also provided the concentration of the RNA solutions.

3.2.6 Reverse transcription and cDNA synthesis

cDNA synthesis was completed using a reverse transcriptase Taqman kit (Applied Biosystems) following manufacturer’s instruction. From 1 µg of RNA, 50 µL of cDNA
was synthesized using 2.5 μL of 50μM random hexamers, and 1.5 μL of 50 U/μL MultiScribe reverse transcriptase in the presence of dNTPs. The reverse transcript reaction was run at 25°C (RT enzyme reaction) for 10 minutes, 48°C (primer annealing) for 30 minutes and 95°C (denaturation) for 5 minutes on a Mastercycler Gradient instrument (Eppendorf).

3.2.7 Quantitative Real Time Polymerase Chain Reaction (RT-PCR) method

The qPCR was run at 60-62°C on an ABI 7900 Fast Sequence Detection instrument (Applied Biosystem) using a syber green detector. The Real Time PCR - SYBR Green master mix (Applied Biosystems) was used. In the total solution of 20 μL, cDNA (0.8 μL) was analyzed for the interested genes (type I collagen (Coll I), osteocalcin (OC) and cbfa-1) and also the reference gene (18S rRNA). 18S rRNA was chosen as the reference gene because of its high stability of expression [252]. The expression of genes of interest under the influence of HA-Fe$_3$O$_4$ nanoparticles were compared to the reference gene and also to the expression of the same genes in the sample without nanoparticles using the $\Delta\Delta C_T$ method [253]. The results were reported as the fold change of gene expression in the tested samples and the control samples (without nanoparticles) (Equation 3.1).

$$\frac{X_{test}}{X_{control}} = 2^{\Delta\Delta C_T} = 2^{(C_{T,X} - C_{T,R})_{control} - (C_{T,X} - C_{T,R})_{test}}$$

(3.1)
where $X_{\text{test}}$ is the threshold number of molecules in tested samples, $X_{\text{control}}$ is the threshold number of molecules in control sample, $C_{T,X}$ is the threshold cycle of the gene of interest and $C_{T,R}$ is the threshold cycle of the endogenous reference gene.

Primers for Coll I, cbfa1, OC, and 18S were designed according to ref [252]. The nucleotide sequences of the primers are shown in Table 3.1.

Table 3.1 Description of designed primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>CGGCTACCACATCCAAGGAA</td>
<td>GCTGGAATTACCGCGGCT</td>
</tr>
<tr>
<td>rRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coll I</td>
<td>CAGCCGCTTCACCTACAGC</td>
<td>TTTGTATTCAATCACTGTCTTGCC</td>
</tr>
<tr>
<td>OC</td>
<td>GAAGCCCAGCGGTGCA</td>
<td>CACTACCTCGCTGCCCTCC</td>
</tr>
<tr>
<td>cbfa1</td>
<td>GCCTTCAAGGTGGTAGCCC</td>
<td>CGTTACCGCCATGACAGTA</td>
</tr>
</tbody>
</table>

3.2.8 Statistical analyses

Results were analyzed using student T-tests and data were expressed as the mean ± standard error of the mean (SEM). Protein adsorption experiments were run in triplicate and gene expression experiments were run in quadruplicate.
3.3 Results and discussion

3.3.1 FTIR spectra of nanoparticles

The FTIR spectra of HA coated Fe$_3$O$_4$ nanoparticle before and after incubation in serum is presented in Figure 3.3. Both spectra showed several characteristic dips of HA. A strong dip at 3570 cm$^{-1}$ and a dip at $\sim$ 651 cm$^{-1}$ were attributed to O-H$^-$ stretching and O-H$^-$ libration, characteristics of HA [254-255]. After incubation in serum, the strength of this dip was reduced, as expected, since the material was coated with serum proteins. The dips at 1083 and 962 cm$^{-1}$ were attributed to the two vibration modes of (PO$_4$)$_3^-$ (Figure 3.4). Two (PO$_4$)$_3^-$ bending modes previously observed at 569 and 603 cm$^{-1}$ [256] were shifted to 586 and 615 cm$^{-1}$. This could be due to the presence of Fe$_3$O$_4$ and citric acid in HA. Interestingly, the signal at 1550 cm$^{-1}$ detected in HA coated Fe$_3$O$_4$ was absent after incubation of the nanoparticle in DMEM with serum. This weak dip was attributed to the carboxylic acid bond, potentially from citric acid. Thus, the proteins in serum might have competed and replaced carboxylic groups on the nanoparticle surface. Although most dips presented before the serum coating also appeared after incubation, three dips at 2964, 2933, 2879 cm$^{-1}$ were only visible for the HA coated Fe$_3$O$_4$ in DMEM. These dips were assigned to the alkyl bonds from serum proteins. Because alkyl is a common hydrocarbon group, it is difficult to identify the specific protein that adsorbed on nanoparticle surfaces from the FTIR result.
Figure 3.3 Wide scan FTIR spectra of HA coated Fe$_3$O$_4$ nanoparticles before (red) and after (blue) incubation in DMEM supplemented with 10% FBS and 1% P/S.

Figure 3.4 Narrow region of the FTIR spectra of HA coated Fe$_3$O$_4$ nanoparticles before (red) and after (blue) incubation in DMEM supplemented with 10% FBS and 1% P/S.
3.3.2 Protein adsorption

Protein adsorption results are presented in Figure 3.5. For both albumin and fibronectin adsorption, there was a trend of increased protein adsorption with higher nanoparticles. However, for albumin adsorption, the increment was gradual. In fact, within the error of the measurements, the albumin adsorbed on the 200 µg/mL HA coated Fe₃O₄ nanoparticles was not significantly higher compared to the 100 and 12.5 µg/mL. In contrast, the fibronectin adsorption on the 200 µg/mL HA coated Fe₃O₄ nanoparticles was significantly higher compared to the fibronectin adsorption on the lower concentration samples. From 12.5 µg/mL to 100 µg/mL, the fibronectin adsorption almost doubled and from 12.5 µg/mL to 200 µg/mL, a 4 fold increment was observed.

Figure 3.5 Protein adsorption on HA coated Fe₃O₄ nanoparticle surfaces. Albumin and fibronectin levels were determined on samples with nanoparticle concentrations of 12.5, 100 and 200 µg/mL. Protein levels were normalized to the level of the lowest nanoparticle concentration (12.5 µg/mL). Data = mean ± SEM. n=3. *p<0.01 compared to fibronectin adsorption on samples with nanoparticle concentrations of 100 and 12.5 µg/mL.
When nanoparticle concentration increased, there was more surface area for protein adsorption. However, the increase in albumin adsorption was very low compared to that of fibronectin. The drastic enhancement in fibronectin adsorption on the HA coated Fe$_3$O$_4$ nanoparticle surface could be attributed to the response of proteins to nano scale surfaces. Webster et al. studied the adsorption of several proteins (such as laminin, albumin, vitronectin and fibronectin) on conventional smooth surfaces (borosilicate glass) and nano scale surfaces of different grain sizes (179 nm and 67 nm grain size hydroxyapatite) [44]. The results showed that while the adsorption of laminin decreased on 67 nm grain size surfaces compared to smooth and 179 nm grain size surfaces, the amount of albumin adsorbed was not different for all three types of surfaces. This means that the response of albumin to surface roughness was not specific. On the other hand, the adsorption of pro-osteoblast proteins, fibronectin and vitronectin, was greatly enhanced on the 67 nm grain surfaces compared to 179 nm and smooth surfaces. This trend was not only observed on nano HA but was also found on nano titania and nano alumina [44]. The results indicated that the amount of protein adsorbed on the nanoparticle surface does not respond linearly to the increase of surface area. This could partially be due to changes in surface charge and wettability, which consequently changed protein folding when adsorbed on particle surfaces.

In the current case, the HA nanoparticle size was 60 nm long and 20 nm wide. This size scale is close to the surface grain size of 67 nm that Webster et al. studied. Therefore, by increasing nanoparticle concentration, the amount of fibronectin adsorbed on nanoparticle surfaces was enhanced. Consequently, when interacting with osteoblasts,
the fibronectin coated nanoparticle provided more surface area for cell adhesion and spreading, which lead to better proliferation and differentiation results mentioned in Chapter 2. Another factor that contributed to improved cell adhesion and function is the binding between RGD sequences in fibronectin with integrins (such as $\alpha 5\beta 1$, $\alpha IIb\beta 3$ and $\alpha V\beta 3$ integrins) on osteoblast membranes.

It is also important to note that although the higher concentration of HA coated Fe$_3$O$_4$ adsorbed higher amounts of fibronectin, the cytotoxicity limit will be reached if we keep increasing nanoparticle concentrations. The limit concentration in this study was 200 $\mu$g/mL and was less than the cytotoxicity limit. Moreover, higher nanoparticle concentrations can facilitate nanoparticle agglomeration and, thus, compensate for the increased surface area for protein adsorption. Further studies should be completed to determine such limits.

### 3.3.3 Osteoblast gene regulation in the presence of nanoparticles

Cell – biomaterial interactions are very complex and involve many processes. At an earlier time point, the interaction involves various biological macromolecules (such as extracellular matrix proteins, cell membrane proteins and serum proteins). These proteins interact to promote signal transduction and transcription factors in cells. Ultimately, these interactions result in gene expression regulation. The current study focused on three genes that are considered important to osteoblasts: type I collagen (Figure 3.6), osteocalcin (Figure 3.7) and cbfa-1 (Figure 3.8).

After the exposure of HA coated magnetic nanoparticles to osteoblasts, all genes studied were normally expressed compared to control samples (no particles added). This
result indicated that after 24 hours of treatment, the nanoparticles did not induce any genotoxicity to osteoblasts but also did not upregulate any of the genes of interest. In Chapter 2, it was demonstrated that the markers (such as collagen synthesis and calcium deposition) did not increase until 21 days of incubation. Therefore, a longer treatment time may be needed to measure the differences in gene expression between nanoparticle treated and non-treated samples.

Figure 3.6 Expression of type I collagen by osteoblasts under the influence of HA coated Fe₃O₄ magnetic nanoparticles. Expression fold changes were normalized to control samples (no nanoparticles). Data = mean ± SEM. n=4.
Figure 3.7 Expression of osteocalcin by osteoblasts under the influence of HA coated Fe$_3$O$_4$ magnetic nanoparticles. Expression fold changes were normalized to control samples (no nanoparticles). Data = mean ± SEM. n=4.

Figure 3.8 Expression of cbfa-1 by osteoblasts under the influence of HA coated Fe$_3$O$_4$ magnetic nanoparticles. Expression fold changes were normalized to control samples (no nanoparticles). Data = mean ± SEM. n=4.
3.4 Conclusions

This chapter of the thesis focused on protein adsorption and bioactivity on nanoparticle surfaces and the expression of three genes (type I collagen, osteocalcin and cbfa1) that are closely related to osteoblast functions. It was shown that proteins in serum adsorbed on and changed the nanoparticle surface. FTIR spectra of the nanoparticles incubated in serum showed exclusive dips compared to that of the original nanoparticle. However, it was difficult to identify the type of proteins from FTIR spectra alone. After adsorption to nanoparticle surfaces, these proteins promoted the response of osteoblasts to the nanoparticles. Adsorption of two specific proteins, albumin and fibronectin, were investigated in more details here. ELISA experiments demonstrated the enhancement of fibronectin adsorption with higher concentrations of HA coated Fe$_3$O$_4$ nanoparticles. As fibronectin is known to improve bone cell adhesion, migration and spreading [235-236], it was observed that the highest amount of fibronectin adsorbed on 200 $\mu$g/mL HA coated Fe$_3$O$_4$, led to better bone cell proliferation and differentiation as demonstrated in Chapter 2.

The gene expression of osteoblasts under the influence of HA coated nanoparticles was also investigated. At the particle concentrations (200, 100 and 12.5 $\mu$g/mL) studied, no genotoxicity was detected. A longer time period of investigation should be completed in the future to determine the difference in gene expression in the presence of these nanoparticles, which may provide further evidence of the molecular recognition of HA coated Fe$_3$O$_4$ nanoparticles.
CHAPTER 4. NANOPARTICLE UPTAKE BY OSTEOBLASTS VIA RECEPTOR-MEDIATED ENDOCYTOSIS

4.1 Introduction of the endocytosis pathway

Endocytosis is a very important process in which cells internalize extracellular materials such as proteins, ligands, nutrients, and particles. It is also the pathway that toxins and viruses enter cells. In general, endocytosis can be divided into phagocytosis (or “cell eating”), pinocytosis (or “cell drinking”) and receptor-mediated endocytosis. Phagocytosis, which is the uptake of large particles (> 500 nm) or bacteria, can take place in many cell types [257]. However, phagocytosis is most important in phagocytic cells such as macrophages or neutrophils since those cells directly involve the uptake and degradation of infectious agents, tissue remodeling, immune responses and inflammation [258]. Pinocytosis is considered as the uptake of fluid and solutes into cells. In contrast to an actin – dependent mechanism of phagocytosis, the pinocytic route is mediated by clathrin, a protein that forms coated vesicles for cell uptake [259].

In this study, we focused on receptor – mediated endocytosis. This endocytosis pathway is particularly important because it is the process that allows nanoparticles and viruses to enter the cells [260]. A diagram of endocytosis pathway is presented in Figure 4.1. As demonstrated in the diagram, a cell has various proteins called receptors distributed on the plasma membrane. These receptors can recognize certain extracellular proteins call ligands. Epidermal growth factor (EGF) receptor, low-density lipoprotein (LDL) receptor and transferrin receptors are demonstrated here. In the absence of ligands, the receptors can be distributed randomly on the cell surface (EGF receptor) or
accumulated in coated pits in the cell membrane (LDL and transferrin receptors). Most of the pits on cell membrane are coated with a protein called clathrin [261]. However, several studies on viruses entering cells demonstrated such process were clathrin independent [262-264].

After ligands bind to surface receptors, the ligand-receptor complexes are clustered in coated pits and are taken into the cell by receptosomes or endosomes. The pH inside endosomes is normally around 4.5 [265]. Therefore some ligands and receptors start to dissociate inside endosomes. The endosomes then fuse with the Golgi system in which the fate of the receptors and ligands are determined. Some ligands and receptors (like transferrin) are returned to the cell surface where ligands are released from the cell and the receptors are reused. Some other ligands end up and degrade in lysosomes, while their receptors return to the membrane surface (e.g. asialoglycoprotein [266]). Finally, other ligands and receptors are directed to lysosomes and degrade there.

As described in Chapter 3, when a nanoparticle is first placed in cell media, its surface is coated with serum proteins. These proteins or peptides in the proteins will act as ligands and interact with specific receptor on cell surface. It was shown that fibronectin adsorption increased on higher concentrations of HA coated Fe_3O_4 nanoparticles (200 µg/mL). In the current study, the RGD peptide in fibronectin adsorbed on nanoparticle surfaces may have acted as a ligand to interact with integrin receptors (such as fibronectin specific integrins: α5β1, αIIbβ3 and αVβ3) on osteoblast surfaces. The nanoparticle then enters the cell in the same way ligands do. However, unlike ligands, many nanoparticles cannot be degraded in lysosome. The lysosome will
eventually fuse with the cell membrane and the nanoparticles exit the cell via a process called exocytosis.

Figure 4.1 A diagram of the endocytosis pathway [266]. The ligands shown are (E) for EGF, (T) for transferrin, and (α) for α2–macroglobulin. The respective receptors are (●) for the EGF receptor, (●) for the transferrin receptor and (●) for the α2–macroglobulin receptor.

4.2 Endocytosis model

So far, several models have been developed to explain the mechanism of receptor-mediated endocytosis [267-270]. Most of these models concentrated on modeling the dynamic of endocytosis and viral budding. Among those models, the work by Gao et al. is notable [271]. Using a mechanical approach, the authors presented a model in which a
single particle with surface immobilized ligands gradually bound to the receptors on the cell surface. Free receptors on the cell membrane then diffused toward the binding site and linked to the ligands there. This process continued until the total number of receptors at the site was equal to the total number of ligands on the particle surface. The particle was then completely engulfed by the cell membrane. The study predicted a size dependent endocytosis rate, which was confirmed later by Jiang et al. [272].

In this study, instead of investigating the behavior of a single nanoparticle, the kinetics of the endocytosis process was considered. The uptake pathway of nanoparticles into a cell is described in Figure 4.2. First, the nanoparticles with ligands immobilized on the surface move and bind to receptors on the plasma membrane. The nanoparticles, ligands and receptors are then taken into the cell via endosomes. At this point, receptors and ligands dissociate and follow two different routes. Receptors return to cell surfaces to be re-utilized while nanoparticles and ligands go through the lysosome, where ligands are degraded. Since the cell is unable to digest the nanoparticles in lysosomes, the nanoparticles are then “pushed out” by a processed called exocytosis. Therefore, the total number of particles inside the cell is dynamic and is the balance between endocytosis and exocytosis.
The current model is based on following assumptions:

- The rate of receptors taken into the cell is \( k_1 \).
- The total number of receptors on the cell surface and inside the cell is always conserved and equal to \( N_0 \). The receptors are continuously recycled at rate \( k_2 \).
- The rate of nanoparticle exocytosis is \( k_3 \).
- \( k_1 \), \( k_2 \) and \( k_3 \) are independent of time, but \( k_1 \) depends on external nanoparticle concentrations (doses).
- At time \( t=0 \), the number of receptors on the surface is equal to \( N_0 \) and the number of nanoparticles inside the cell is equal to 0.
Using the above assumptions, the number of surface receptors as a function of time will be established. Based on the number of surface receptors, the number of nanoparticles inside a cell will be calculated. The dynamics of endocytosis will then be investigated using the relationships among $k_1$, $k_2$ and $k_3$.

4.2.1 Number of surface receptors

First, the number of receptors on the cell membrane, $N$, is calculated. The rate of receptor endocytosis is given by:

rate of receptor endocytosis = $k_1 \times N$

and the rate of recycling is:

rate of recycling = $k_2 \times (N_o - N)$

Therefore, the rate of change of the number of receptors on the surface is:

$$\frac{dN(t)}{dt} = -k_1 N + k_2 (N_o - N)$$  \hspace{1cm} (4.1)

Thus,

$$\frac{dN}{dt} + (k_1 + k_2)N = k_2 N_o$$  \hspace{1cm} (4.2)

which leads to:

$$N(t) = \frac{k_2}{(k_1 + k_2)} N_o + C_o e^{-(k_1 + k_2)t}$$  \hspace{1cm} (4.3)

where $C_o$ is a constant. Apply the initial condition for $N$, we have:

$$C_o = \frac{k_1}{(k_1 + k_2)} N_o$$  \hspace{1cm} (4.4)
Therefore, the number of surface receptors is:

\[
N(t) = \frac{k_2}{(k_1 + k_2)} N_o + \frac{k_1}{(k_1 + k_2)} N_o e^{-(k_1 + k_2)t} \tag{4.5}
\]

### 4.2.2 Number of nanoparticles inside the cell

The rate of nanoparticle endocytosis is \( \frac{k_1}{\alpha} \times N \), where \( \alpha \) is the number of ligands per nanoparticle. Therefore, the rate of change of the number of nanoparticles inside the cell, \( N_p \), is given by:

\[
\frac{dN_p(t)}{dt} = \frac{k_1}{\alpha} N - k_3 N_p \tag{4.6}
\]

Substituting equation 4.5 into equation 4.6, we have:

\[
\frac{dN_p(t)}{dt} = \frac{k_1 k_2}{\alpha(k_1 + k_2)} N_o + \frac{k_1^2}{\alpha(k_1 + k_2)} N_o e^{-(k_1 + k_2)t} - k_3 N_p
\]

Hence,

\[
\frac{dN_p(t)}{dt} + k_3 N_p = \frac{k_1 k_2}{\alpha(k_1 + k_2)} N_o + \frac{k_1^2}{\alpha(k_1 + k_2)} N_o e^{-(k_1 + k_2)t}
\]

Therefore:

\[
\frac{d}{dt} (N_p e^{k_3 t}) = \left[ \frac{k_1 k_2}{\alpha(k_1 + k_2)} N_o + \frac{k_1^2}{\alpha(k_1 + k_2)} N_o e^{-(k_1 + k_2)t} \right] e^{k_3 t}
\]

\[
(4.9)
\]
After integration, equation 4.9 becomes:

\[
N_p = \frac{k_1 k_2}{\alpha k_3 (k_1 + k_2)} N_o + \frac{k_1^2}{\alpha (k_1 + k_2) (k_3 - k_1 - k_2)} N_o e^{-(k_1 + k_2) t} + C_1 e^{-k_3 t}
\]

(4.10)

Applying the initial condition for the number of nanoparticles inside the cell, 
\( N_p(t = 0) = 0 \) into equation 4.10, we have:

\[
C_1 = -\frac{k_1 N_o}{\alpha (k_1 + k_2)} \left( \frac{k_2}{k_3} + \frac{k_1}{k_3 - k_1 - k_2} \right)
\]

(4.11)

and therefore, \( N_p \) is:

\[
N_p(t) = \frac{k_1 k_2 N_o}{\alpha k_3 (k_1 + k_2)} + \frac{k_1^2 N_o}{\alpha (k_1 + k_2) (k_3 - k_1 - k_2)} e^{-(k_1 + k_2) t} - \frac{k_1 N_o}{\alpha (k_1 + k_2)} \left( \frac{k_2}{k_3} + \frac{k_1}{k_3 - k_1 - k_2} \right) e^{-k_3 t}
\]

(4.12)

According to equation 4.12, the total number of nanoparticles inside the cell is completely calculated if \( k_1, k_2, k_3, \alpha \), and \( N_o \) are known. The behavior of \( N_p(t) \) is governed by the two exponential functions \( e^{-(k_1 + k_2) t} \) and \( e^{-k_3 t} \).

It should be noted that when \( t \to \infty \), the two above exponential functions go to zero and \( N_p \) is a constant:

\[
N_p(t) = \frac{k_1 k_2 N_o}{\alpha k_3 (k_1 + k_2)}
\]

(4.13)

Equation 4.13 indicates that the total number of nanoparticles inside the cell due to endocytosis starts at zero and saturates as time increases.
4.2.3 The behavior of the number of nanoparticles inside the cell: $N_p(t)$

Due to our definition, $k_1$, $k_2$ and $k_3$ are all positive real numbers. Based on the relationship of $k_1$, $k_2$ and $k_3$ in equation 4.12, we consider three following cases:

(a) $k_3 > k_1 + k_2$

In this case, the 2nd term on the right hand side of equation 4.12 is positive and the 3rd term is negative. The second exponential, $e^{-k_3 t}$ will dominate at the earlier time points. Therefore, $N_p$ will increase at first, then decreases to reach saturation value in equation 4.13.

(b) $k_3 = k_1 + k_2$

In this case, equation 4.9 is reduced to:

$$\frac{d}{dt}(N_p e^{k_3 t}) = \frac{k_1 k_2}{\alpha(k_1 + k_2)} N_o e^{k_3 t} + \frac{k_1^2}{\alpha(k_1 + k_2)} N_o$$

(4.14)

and therefore,

$$N_p(t) = \frac{k_1 k_2 N_o}{\alpha k_3 (k_1 + k_2)} (1 - e^{-k_3 t})$$

(4.15)

Equation 4.15 indicates that $N_p$ will increase continuously to reach a saturation value in equation 4.13. The uptake half-life can be calculated directly from $k_3$:

$$T = \frac{ln2}{k_3}$$

(4.16)

(c) $k_3 < k_1 + k_2$

In this case, the 2nd term on the right hand side of equation 4.12 is negative.

The sign of the 3rd term is dependent on $\frac{k_2}{k_3} + \frac{k_3}{k_3 - k_1 - k_2}$.

If $\frac{k_2}{k_3} + \frac{k_1}{k_3 - k_1 - k_2} > 0$, we have:
\(-k_2k_3 + k_1k_2 + k_2^2 > k_1k_3\)  \hspace{1cm} (4.17)

\[k_1k_2 + k_2^2 > k_1k_3 + k_2k_3\]  \hspace{1cm} (4.18)

\[k_2 > k_3\]  \hspace{1cm} (4.19)

The 3rd term on the right hand side of equation 4.12 is also negative. Therefore, \(N_p\) will increase continuously to reach saturation value in equation 4.13.

If \(\frac{k_2}{k_3} + \frac{k_1}{k_3-k_1-k_2} < 0\), we have:

\[k_2 < k_3 < k_1 + k_2\]  \hspace{1cm} (4.20)

The 3rd term on the right hand side of equation 4.12 is positive. The first exponential, \(e^{-(k_1+k_2)t}\), will dominate at the earlier time points. Therefore, \(N_p\) will increase at first, then decrease to reach saturation value in equation 4.13.

From this endocytosis model, if \(k_1, k_2, k_3, \alpha\), and \(N_o\) are known, the number of receptors on the plasma surface and also the number of nanoparticles inside the cell can be calculated. However, the experimental data of these parameters are largely unknown [267]. Therefore, the proposed model is not intended to mimic the osteoblast uptake in this study. Nevertheless, the calculation using the model will take into account parameters found in several other studies on different systems. The results will help explain the behavior of the receptor – mediated endocytosis pathway.

4.3 Materials and methods
To study nanoparticle uptake into cells, osteoblasts (CRL-11372, ATCC, population number 9) were seeded at 34,000 cells/cm² and cultured in DMEM supplemented with 10% FBS and 1% P/S under standard conditions (37°C, humidified, 5% CO₂, 95% air environment) for 12 hours. This seeding density ensured that osteoblasts formed a monolayer on the bottom of the culture plate and, thus, reduced unspecific binding of nanoparticles to the plate. After the incubation period, Fe₃O₄ and HA coated Fe₃O₄ nanoparticles, which were synthesized as described in Chapter 2, were added to the cell solutions at the concentrations of 200, 100 and 12.5 µg/mL. The cells were again incubated at 37°C in a humidified, 5% CO₂, 95% air environment. At time points of 1, 2.5, 4, 7 and 10 hours after the addition of the magnetic nanoparticles, osteoblasts were rinsed with PBS for three times before being digested using 2mL of aqua regia. Then, the aqua regia was heated at 300°C and evaporated using a hot plate. Resulting salts were dissolved in 5 mL of HNO₃ 2% solution (Sigma) for elemental analysis using the inductively coupled plasma (ICP) technique (JY2000 Ultrace ICP Atomic Emission Spectrometer - Horiba). Two wells containing cells without nanoparticles were treated in the same way and were considered as control samples. The measured amounts of Fe and Ca in control samples were subtracted by the total amount of Fe and Ca in the wells of interest to obtain the uptake of Fe and Ca into osteoblasts. The measured uptake amounts were converted to number of nanoparticles and normalized to the total number of cells. The conversions were based on the sizes of Fe₃O₄ and HA coated Fe₃O₄ nanoparticles, which were reported in Chapter 2. A Fe₃O₄ nanoparticle was considered as a sphere with a radius of 10 nm and a HA coated Fe₃O₄
nanoparticle was a cylinder with a height of 60 nm and a base radius of 10 nm. The density of Fe₃O₄ and HA was 5,000 kg/m³ [273] and 3,156 kg/m³ [274], respectively.

Since receptor-mediated endocytosis is a temperature dependent process [266], the same experiment was conducted at 4°C for osteoblasts in the presence of Fe₃O₄ and HA coated Fe₃O₄ at a concentration of 200 µg/mL. The uptake percentages at 4°C were then compared to the ones at 37°C.

Results were analyzed using Student t-tests and data were expressed as the mean ± standard deviation (SD). The experiments were run in duplicate.

4.4 Results and discussion

4.4.1 Nanoparticle uptake by osteoblasts

The uptake of Fe₃O₄ nanoparticles by osteoblasts is reported in Figure 4.3. At two particle concentrations studied (200 µg/mL and 100 µg/mL), the nanoparticle uptake amount saturated after 6 to 7 hours. Specifically, the uptake half-life for the sample added with 200 µg/mL and 100 µg/mL of Fe₃O₄ were 2.5 and 2.4 hours, respectively. The sample with 12.5 µg/mL of Fe₃O₄ showed a continuous increase in cell uptake. The results also showed that the uptake amounts depended on the initial concentration of the nanoparticles. With a higher concentration of nanoparticle solution added, more nanoparticles were found inside osteoblasts. For samples added with a 200 µg/mL Fe₃O₄ solution, the saturation value was 2.75 billion nanoparticles per cell and for samples added with a 100 µg/mL Fe₃O₄ solution, the number was 1.2 billion nanoparticles per cell.
Figure 4.3 Time dependent uptake of Fe$_3$O$_4$ nanoparticles by osteoblasts. Data = mean ± SD. n=2. * p<0.05 compared to samples at the same time point but added at lower concentrations of nanoparticles. ** p<0.05 compared to the same samples at earlier time points.

The uptake of HA coated Fe$_3$O$_4$ nanoparticles by osteoblasts is reported in Figure 4.4. The endocytosis trend was similar to the uptake of Fe$_3$O$_4$ nanoparticles. The number of HA coated Fe$_3$O$_4$ nanoparticles inside the cell increased with time and finally reached saturation. At all three particle concentrations studied (200 µg/mL, 100 µg/mL and 12.5 µg/mL), the nanoparticle uptake amount saturated after 3 to 4 hours. Specifically, the uptake half-life for samples added with 200 µg/mL, 100 µg/mL and 12.5 µg/mL of HA coated Fe$_3$O$_4$ were 1.5, 2.4 and 2.5 hours, respectively. Compared to the case of Fe$_3$O$_4$ nanoparticles, the HA coated nanoparticle uptake amounts increased more rapidly, which reflected the shorter uptake half-life. Interestingly, the number of HA coated Fe$_3$O$_4$ nanoparticles decreased at the 7 hours time point, which was not observed in the case of the Fe$_3$O$_4$ nanoparticle uptake. Since the experiments were only run in duplicate, these
reductions could result from experimental errors. However, this result could also be due to the decreases that were pointed out in section 4.2.3 of the endocytosis model.

The results also showed that the uptake amounts depended on the initial concentration of the nanoparticles. With a higher concentration of nanoparticle solution added, more nanoparticles were found inside osteoblasts. For samples added with a 200 µg/mL, 100 µg/mL and 12.5 µg/mL of HA coated Fe₃O₄ solution, the saturation numbers were 10, 5.8 and 1.6 billion nanoparticles per cell.

![Figure 4.4](image.png)

Figure 4.4 Time dependent uptake of HA coated Fe₃O₄ nanoparticles by osteoblasts. Data = mean ± SD. n=2. * p<0.05 compared to samples at the same time point but added with lower concentrations of nanoparticles. ** p<0.05 compared to the same samples at earlier time points.

Due to the small size of nanoparticles (less than 100 nm), their internalization into the cells was generally receptor – mediated endocytosis. Previous studies by Chithrani et al. on the endocytosis route of gold nanoparticles into HeLa cells also showed the same saturation uptake trend [275]. The uptake half-life for 14 nm and 74 nm gold nanoparticles were 2.1 and 2.24, respectively. The same group later identified that the
endocytic route was indeed receptor-mediated endocytosis by confirming decreased cell uptake at 4°C [276]. Using sucrose and potassium depletion treatments to disrupt clathrin-coated vesicle formation, the endocytosis pathway was verified to be clathrin dependent. Therefore, temperature dependent cellular uptake for Fe₃O₄ and HA coated Fe₃O₄ nanoparticle experiments were completed and are presented as follow.

4.4.2 Receptor-mediated endocytosis at low temperatures

Receptor-mediated endocytosis involves a complex process in which the unbound receptors on the plasma membrane diffuse toward the ligand binding site to facilitate particle wrapping [277]. At low temperatures, the movements of surface receptors are inhibited [278]. Therefore, the nanoparticles can bind to surface receptors but cannot be internalized. In the current study, nanoparticle uptake experiments were completed at 4°C to compare to the endocytosis at 37°C. Cellular uptake experimental results at 4°C and 37°C using osteoblasts supplemented with a 200 µg/mL solution of Fe₃O₄ nanoparticles are shown in Figure 4.5. Similar to the saturation uptake at 37°C, the endocytosis of Fe₃O₄ nanoparticles was time dependent. However, the saturation value of nanoparticle uptake at 4°C decreased 25% compared to the saturation uptake at 37°C. Because of the setup of the experiments, the particles that bound to the cell surface were also included. Therefore, nanoparticle endocytosis was not completely shut down at 4°C. A transmission electron microscopic image of the cell incubated with nanoparticle at 4°C can confirm the inhibition of receptor-mediated endocytosis at 4°C. There is also a chance that the nanoparticles enter the cell via another process not involving receptors (such as phagocytosis) and, thus, is not limited by low temperature.
Figure 4.5 Endocytosis of Fe$_3$O$_4$ nanoparticles at 37°C and 4°C. Data = mean ± SD. n = 2. *p<0.05 compared to the uptake percentage at 4°C after 10 hours.

HA coated Fe$_3$O$_4$ nanoparticle uptake was also inhibited when the cells were cultured at 4°C in the presence of 200 µg/mL nanoparticles (Figure 4.6). Specifically, at 10 hours, the uptake percentage at 4°C was 47% less than that amount of endocytosis at 37°C. In Chapter 2, it was shown that HA coated Fe$_3$O$_4$ nanoparticles were internalized as large aggregates (~500 nm). The current result emphasized that the endocytosis route of HA coated nanoparticles was also receptor dependent. Therefore, the uptake mechanism of HA coated nanoparticles into osteoblasts could be attributed to both phagocytosis and receptor – mediated endocytosis.
Figure 4.6 Endocytosis of HA coated Fe$_3$O$_4$ nanoparticles at 37°C and 4°C. Data = mean ± SD. n = 2. *p<0.05 compared to the uptake percentage at 4°C after 10 hours.

The temperature dependent cellular uptake results confirmed that Fe$_3$O$_4$ and HA coated Fe$_3$O$_4$ nanoparticles were internalized into osteoblasts via receptor – mediated endocytosis. However, whether there were additional endocytosis pathways was unknown. It was also not clear that the endocytosis pathway was clathrin dependent or not. Further studies using sucrose and potassium depletion methods can clarify the mechanism of the pathway.

4.4.3 Receptor – mediated endocytosis model

Using equation 4.5, the number of receptors on the cell surface was calculated and normalized to the total number of receptors at $t = 0$, $N_0$. The number of surface receptors only depend $k_1$ and $k_2$ but not $k_3$. Figures 4.7 and 4.8 show the theoretical predictions of the total number of receptors on the surface with respect to time. In Figure 4.7, $k_1$ was fixed and $k_2$ varied. $k_1$ was chosen to equal 0.6 hr$^{-1}$. When $k_2$ was small, the receptor
recycling rate was low and thus the number of receptors on the surface decreased 85% and reached saturation. When the recycling rate increased, the saturation number of the surface receptors also increased. Also, when $k_2$ increased, the half-life of the receptor reduction reduced, which meant that the number of receptors reached saturation quicker. The coefficients were chosen according to the reference [279].

Figure 4.7 Relative number of surface receptors as a function of time. $k_1$ was fixed and $k_2$ varied.

In the calculation described in Figure 4.8, $k_2$ was fixed at 0.6 hr$^{-1}$ and $k_1$, the endocytosis rate, varied. At a smaller uptake rate, the receptors were taken into the cell slowly compared to the higher rate of receptor recycling. Therefore, the total number of surface receptors only dropped 15% to reach saturation. The half-life of this reduction was longer compared to the half-life with $k_1 = 0.4$, 1 and 10 hr$^{-1}$. When $k_1$ increased, the uptake rate of surface receptor increased, which led to the reduction in saturation number of surface receptors. For $k_1 = 10$ hr$^{-1}$, a dramatic drop was observed due to the
combination of the low saturation number of surface receptors and short half-life of the exponential reduction.

Figure 4.8 Relative number of surface receptors as a function of time. $k_2$ was fixed and $k_1$ varied.

Next, using equation 4.12, the total number of nanoparticles inside the cell was calculated and normalized to $\frac{N_0}{\alpha}$. In the calculations, $k_1$ and $k_2$ were fixed at 0.6 hr$^{-1}$ and $k_3$ varied. The behavior of the number of nanoparticles with respect to time was justified based on the relationship among $k_1$, $k_2$ and $k_3$.

Figure 4.9 shows the relative nanoparticle uptake when $k_3 > k_1 + k_2$. The relative uptake was calculated for $k_3 = 1.3, 3$ and 10 hr$^{-1}$. For $k_3 > k_1 + k_2$, the uptake curve increased at first then decreased and reached saturation. From equation 4.13, the saturation value of uptake increased as $k_3$ decreased. The behavior of relative uptake as a function of time resembled the endocytosis of HA coated Fe$_3$O$_4$ nanoparticles described in section 4.3.1.
Figure 4.9 Relative number of surface receptors as a function of time. $k_1$ and $k_2$ were fixed at 0.6 hr$^{-1}$ and $k_3$ varied. The figure shows calculations for $k_3 = 1.3$, 3 and 10 hr$^{-1}$.

When $k_2 < k_3 < k_1 + k_2$, the $3^{rd}$ term on the right hand side of equation 4.12 was positive. Therefore, the exponential, $e^{-(k_1+k_2)t}$, dominated at the earlier time points. Consequently, similar to the case when $k_3 > k_1 + k_2$, $N_p(t)$ increased at first, then decreased to reach a saturation value in equation 4.13. The calculated results for $k_3 = 0.75$, 9 and 1.15 hr$^{-1}$ are shown in Figure 4.10.
Figure 4.10 Relative number of surface receptors as a function of time. $k_1$ and $k_2$ were fixed at 0.6 hr$^{-1}$ and $k_3$ varied. The figure shows calculations for $k_3 = 0.75$, 9 and 1.15 hr$^{-1}$.

When $k_2 < k_3$, the 2$^{nd}$ and the 3$^{rd}$ term on the right hand side of equation 4.12 were both negative. The model predicted that $N_p$ will increase without any reduction and reach a saturation value indicated in equation 4.13. The calculated results are shown in Figure 4.11 for $k_3 = 0.5$, 0.3 and 0.2 hr$^{-1}$. The behavior of the relative uptake amount resembled the endocytosis of Fe$_3$O$_4$ nanoparticles into osteoblasts in Figure 4.3.
Figure 4.11 Relative number of surface receptors as a function of time. $k_1$ and $k_2$ were fixed at 0.6 hr\(^{-1}\) and $k_3$ varied. The figure shows calculations for $k_3 = 0.5$, 0.3 and 0.2 hr\(^{-1}\).

In the current study, due to the lack of detailed information regarding important parameters, it was difficult to develop a quantitative model for the endocytosis pathway of nanoparticles into cells. However, the calculated results provided some insights to the relationships of the endocytosis rate, receptor recycling rate and exocytosis rate. The model showed that the uptake amount of nanoparticles had a saturated trend. In the two nanoparticle systems studied, the Fe\(_3\)O\(_4\) nanoparticle uptake exhibited the behavior similar to when exocytosis rate was lower than receptor recycling rate ($k_3 < k_2$). On the other hand, the uptake of HA coated Fe\(_3\)O\(_4\) nanoparticles with the reduction at the time point 7 hours followed the case when the exocytosis rate was greater than receptor recycling rate.

It is important to note that the uptake of Fe\(_3\)O\(_4\) and HA coated Fe\(_3\)O\(_4\) were both dose dependent. In this current model, $k_2$ and $k_3$ were considered intrinsic values of the
cell and did not depend on initial nanoparticle concentrations. \( \alpha \) and \( N_0 \) were also constants. Therefore, from equation 4.13, the saturation value of uptake was dose dependent only if \( k_1 \) was a function of nanoparticle concentration outside of the cells.

### 4.5 Conclusions

In this study, nanoparticle uptake by receptor – mediated endocytosis was investigated. A theoretical model of endocytosis was developed to understand the mechanism of the internalization of \( \text{Fe}_3\text{O}_4 \) and HA coated \( \text{Fe}_3\text{O}_4 \) nanoparticles into osteoblasts. The cell uptake experiments were completed at time points 1, 2.5, 4, 7 and 10 hours. The results demonstrated a time dependent nanoparticle uptake by osteoblasts. The uptake of HA coated and non coated nanoparticles into osteoblasts had a similar trend and all were saturated after 3 to 4 hours for HA coated \( \text{Fe}_3\text{O}_4 \) and 6 to 7 hours for \( \text{Fe}_3\text{O}_4 \) nanoparticles. The cellular uptake amounts were also dose dependent. The saturation amounts increased when the concentrations of nanoparticle solutions supplemented to the cell increased. To confirm that the endocytic pathway was receptor – mediated, uptake experiments were conducted at 4\( ^\circ \)C to compare to normal uptake at 37\( ^\circ \)C. At lower temperatures, the diffusion of surface receptors was limited and, thus, endocytosis was inhibited. The results of the study showed that after 10 hours of incubation, the uptake percentage of \( \text{Fe}_3\text{O}_4 \) and HA coated \( \text{Fe}_3\text{O}_4 \) at 4\( ^\circ \)C decreased 25\% and 47\%, respectively, compared to the uptake at 37\( ^\circ \)C. Therefore, it was concluded that the endocytosis pathway in the study was at least in part receptor – mediated. However, the study did not clarify if the endocytosis pathway was clathrin dependent or independent. Further investigations using sucrose and potassium depletion methods will elucidate the mechanism of this endocytosis pathway.
The developed model predicted the saturation uptake trend in all nanoparticles. From the relationship among the endocytosis rate, the receptor recycling rate and the exocytosis rate, two different types of saturation curves were observed. When $k_3 > k_2$, the uptake amount increased over the saturation amount and then decreased and reached saturation. This curve resembled the behavior of HA coated Fe$_3$O$_4$ nanoparticle uptake. When $k_3 < k_2$, the uptake amount gradually increased toward saturation without any reduction, which was similar to the uptake curve of Fe$_3$O$_4$ nanoparticles. Further studies on the relation of $k_1$ and nanoparticle doses are necessary to understand the concentration dependent manner of endocytosis. More experimental data are also needed for the model to mimic the current system. Parameters such as the number of ligands on the nanoparticle surface, the number of receptors on cell membrane, the exocytosis rate and the recycling rate are priorities in future studies.
CHAPTER 5. BACTERICIDAL EFFECT OF IRON OXIDE NANOPARTICLES

5.1 Introduction

Bacterial infection causes numerous problems in orthopedic medical devices applications. A recent report showed that bacterial infection was among the top three most common causes of total hip arthroplastic revisions (contributing 14.8% to all failures) [280]. *Staphylococcus aureus* (*S. aureus*) is one of the most common human pathogens that leads to many types of infection [281]. This bacterium is responsible not only for local infections, such as wound or postoperative infections, but also for prosthetic infection (such as catheters, endotracheal tubes and other biomaterials) [282-284]. *S. aureus* is also known to possess an increasing ability to resist antibiotics [285-287] (such as penicillin, methicillin, tetracycline, erythromycin and vancomycin). Thus, it is necessary to find alternative treatments (perhaps without the use of antibiotics) for *S. aureus* infections that are more directed, localized and difficult for bacteria to formulate a resistance.

Along this line, some have hypothesized that reactive oxygen species (ROS) generated by Fe$_3$O$_4$ nanoparticles could kill bacteria without harming non-bacteria cells [288]. Specifically, Pareta et al. cultured osteoblasts (bone forming cell) with iron oxide (IO) nanoparticles (at a concentration of 4.25 mg/mL) and found that cell density was greatly enhanced in the presence of iron oxide nanoparticles compared to cells cultured without nanoparticles [79]. In general, as synthesized non-coated IO nanoparticles form aggregates [111]. Therefore, in this study, the nanoparticles were synthesized with polyvinyl alcohol to enhance particle stability.
Polyvinyl alcohol (PVA) is a polymer commonly used in biomedical applications due to its biocompatibility [289]. For example, PVA has been used to produce PVA-DNA nanoparticles for gene delivery [290] or PVA/poly乳酸 (PLA)/Fe₃O₄ nanoparticle contrast agents for magnetic resonant imaging (MRI) applications [85]. Pure PVA showed no antibacterial properties [291]. Iron oxide nanoparticles stabilized by PVA have been developed in several studies with a particular interest in targeted drug delivery [105, 292-293]. In this study, PVA was used as a matrix to deliver non-agglomerated nanoparticles as suggested by previous researchers [294-295]. The objective of this current study was thus to (i) design, synthesize and characterize polyvinyl alcohol (PVA)/IO nanoparticles and (ii) investigate the bactericidal activity of such nanoparticles when cultured with *S. aureus*.

5.2 Materials and methods

5.2.1 Iron oxide/polyvinyl alcohol (PVA) nanoparticle synthesis

IO nanoparticles were synthesized in situ via a matrix mediated method using PVA similar to previously suggested processes [296-297]. An aqueous solution of PVA (Acros Organics) was mixed with an equal volume of a ferrous/ferric aqueous solution at ambient conditions. The iron-loaded PVA gels were soaked in a stoichiometric amount of a warm aqueous solution of NaOH. The resulting ferrosferric hydroxide dehydrates to give brownish precipitate in the polymer solution.
5.2.2 Characterization of iron oxide/PVA

A droplet of IO nanoparticles was placed on a TEM copper grid and allowed to dry for 10 minutes. The imaging was carried out at 100kV on a Phillips EM420 TEM and size calculations were carried out with ImageJ version 1.42q.

To measure the hydrodynamic size of the nanoparticles, 100 µL of the particle solution was diluted with 1.5 ml of water and placed into a cuvette of a Zetasizer-nano instrument (Malvern). Experiments were conducted in triplicate to obtain an average number-size distribution. The same set up was used to measure Zeta potential.

X-Ray diffraction (XRD) of dried nanoparticle powders of was performed on a Siemens D500 within a 2θ range of 20-80 degrees using Cu Kα radiation. The XRD patterns were compared to magnetite (Fe₃O₄) and also maghemite (γ-Fe₂O₃) standards.

The magnetic properties of the dried nanoparticles were obtained using vibrating sample magnometry (VSM, LakeShore 7040) at room temperature.

5.2.3 S. aureus culture

S. aureus were obtained in frozen form from ATCC (ATCC 25923). The bacteria were thawed on ice for 20 minutes before being plated on an agar plate. The plate was left dried before incubation for 16 hours in a standard cell culture environment (37°C, 5% CO₂, and 95% air). A single colony of S. aureus was selected using a 10 µl loop (Sigma) and inoculated into centrifuge tubes containing 5 ml of tryptic soy broth (Sigma). Bacteria in centrifuge tubes were then incubated at 37°C under agitation at 200 rpm for another 16 hours. At that point, the bacteria solution was diluted in tryptic soy broth to an
optical density of 0.52 at 562 nm using a microplate reader (SpectraMax300, Molecular Devices). According to a standard curve correlating bacteria number with optical density, this value was equivalent to $5 \times 10^6$ cells/mL. The cells were further diluted in tryptic soy broth to $5 \times 10^4$ cells/mL before being added to a new centrifuge tube at 3mL/tube.

Concentrated IO/PVA nanoparticles in water were added to bacteria tubes at different doses (30 µg/mL (low dose), 300 µg/mL (medium dose), and 3 mg/mL (high dose)). A tube of bacteria without nanoparticles served as a control. The IO solution was also added to tubes containing only tryptic soy broth at the same concentration as above and served as a particle control. Bacteria were then incubated under agitation at 200 rpm, 37°C using a shaking incubator (New Brunswick Scientific) for 4 hours, 12 hours and 24 hours before a 200 µL bacteria solution was transferred to a 96-well plate for optical density readings at 562 nm using a microplate reader.

5.2.4  S. aureus live/dead assay

After 4, 12 and 24 hours of incubation, 100 µL of the bacteria suspension was transferred to a 96-well plate (Corning). A Live/Dead assay was performed according to manufacturer’s instructions (Live/Dead BacLight, Invitrogen L7007). Briefly, two solutions containing SYTO 9 dye (Invitrogen L7007) and Propidium idodide (Invitrogen L7007) were mixed and diluted with double distilled water before being added to a bacteria solution at 100 µL/well. The plate was incubated at room temperature in the dark for 15 minutes. Fluorescence intensities for live cells (excitation: 485 nm, emission: 530 nm) and dead cells (excitation: 485 nm, emission: 630 nm) were measured using a
fluorescence microplate reader (SpectraMax M5, Molecular Devices). The two intensities were divided and reported as the ratio of Live/Dead bacteria.

5.2.5 Nanoparticle uptake by osteoblasts and bacteria

*S. aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 25668) were separately incubated with 0.1mg/mL iron oxide nanoparticle solutions to study nanoparticle uptake. The bacteria were grown in tryptic soy broth under standard conditions (37°C, humidified, 5% CO₂/95% air environment). After 1 day, the bacteria were collected and fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1M sodium cacodylate and 0.1M sucrose pH 7.4 buffer (Electron Microscopy Sciences) for 1 hour. To enhance contrast of the images, 1% osmium tetroxide (Electron Microscopy Sciences) was used. After samples were washed 3 times with 0.1M sodium cacodylate and 0.1M sucrose pH 7.4 buffer, the cell solution was dehydrated using a series of ethanol solutions (Fisher) at 25%, 50%, 75%, 95% and 100%. Bacteria were centrifuged to form hard pellets before transferring them into epoxy resins (Electron Microscopy Sciences). The resins were cured in an oven at 60°C for 18 hours. Samples were cut using a microtome and placed on copper grids (Electron Microscopy Sciences). Imaging was carried on a Phillips EM 410 TEM.

5.2.6 Statistical analyses

All experiments were conducted in triplicate and repeated at least three times. Differences between means were determined using a Student’s t-test.
5.3 Results and discussion

5.3.1 Nanoparticle synthesis and characterization

Transmission electron microscopy images of the synthesized PVA coated IO nanoparticles (Figure 5.1) showed that the size of the nanoparticles were 9 nm ± 4 nm. The nanoparticles formed necklace-like chains with a typical length of approximately 100-200 nm. The similar formation was reported in an earlier study, in which IO nanoparticles were believed to precipitate along the polymer chain of PVA [295]. The nanoparticle solution was a clear brownish color with no major type of aggregation visible after months from synthesis when stored at room temperature.

Figure 5.1 Transmission electron microscope images of the synthesized IO/PVA nanoparticles. Scale bar = 20 nm.
The hydrodynamic diameter measurement results (Figure 5.2) showed that with the PVA coating, the IO chain-like particles had an average size of 140 nm.

Figure 5.2 IO nanoparticle size distribution as measured by dynamic light scattering.

The measured average zeta potential was -19.09 mV (Figure 5.3). This low value of zeta potential suggested that the nanoparticle solution was stable mostly due to steric repulsion but not electrostatic repulsion. The long hydrophilic chains of PVA formed association with water molecules thus preventing agglomeration.
Figure 5.3 Zeta-potential of IO nanoparticles in water was measured by a Malvern Zetasizer-nano instrument. The average zeta-potential was -19.09 mV.

The X-ray diffraction pattern confirmed that the final production was a mixture of Fe$_3$O$_4$ and γ-Fe$_2$O$_3$ (Figure 5.4). The existence of γ-Fe$_2$O$_3$ was expected and was due to the oxidation of Fe$_3$O$_4$ during synthesis [295].

Figure 5.4 XRD results of the synthesized nanoparticles confirmed the existence of magnetite (Fe$_3$O$_4$) and maghemite γ-Fe$_2$O$_3$. 

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According to the VSM results (Figure 5.5), the negligible coercivity of IO nanoparticles showed properties of superparamagnetic materials, meaning that these nanoparticles will not retain any magnetism after removal of a magnetic field. The high magnetization and superparamagnetic properties were highly desirable for biomedical applications since larger magnetic particles form aggregates after exposure to a magnetic field. The saturation magnetization of the synthesized IO nanoparticles was 15 emu/g. As expected, due to the PVA coating saturation magnetization of the nanoparticles was lower than that of the bulk phase magnetite (~ 90 emu/g). This value was also slightly lower than saturation magnetization of Fe₃O₄ nanoparticles synthesized in chapter 2 (40 emu/g).

Figure 5.5 Magnetization curves of IO/PVA nanoparticles as measured by VSM at room temperature. The results clearly showed superparamagnetic behavior of the nanoparticles.
5.3.2 Bactericidal activity of PVA coated iron oxide nanoparticles

After 4 hours of incubation, the optical density measurements (Figure 5.6) showed that there was no significant difference in bacteria numbers in the presence of IO nanoparticles. After 24 hours, the optical densities of samples with medium and high concentrations of IO were significantly higher than that of the control samples. However, and more importantly, the results from the live/dead assay demonstrated that after 4 hours, the ratio of Live/Dead bacteria was significantly lower in the solution with the highest dose (3mg/mL) of IO nanoparticles compared with the control sample as well as the low (30 µg/mL) and medium dose (300 µg/mL) samples; the same trend was observed after 12 hours and 24 hours (Figure 5.7). The ratio of Live/Dead bacteria also decreased at longer time points. When incubated with highest dose of IO nanoparticles, the ratio was 40 at 4 hour time point and reduced to 20 at 24 hour time point. The optical density of samples with nanoparticles did not necessarily describe the number of bacteria in the solution. It could be that proteins, such as Eap (extracellular adherence protein) [298], secreted by the bacteria accelerate nanoparticle agglomeration.
Figure 5.6 Optical density reading of *S. aureus* in IO/PVA solution after 4 hours, 12 hours and 24 hours. The results are mean ± SEM. (N=3). Nanoparticle doses were: 30 µg/mL (low dose), 300 µg/mL (medium dose), and 3 mg/mL (high dose). All densities are significantly (*p<0.01) greater at 12 and 24 hours compared to the 4 hours time point.
Figure 5.7 Live/Dead assay results showed lower live/dead ratios in presence of the high dose IO/PVA nanoparticle solution after incubation for (a) 4 hours, (b) 12 hours, and (c) 24 hours. Nanoparticle doses were: 30 µg/mL (low dose), 300 µg/mL (medium dose), and 3 mg/mL (high dose). Data = mean ± SEM; N = 3. * p <0.05 compared to control samples and samples with low and medium doses of IO nanoparticles.

There are several factors that caused the presently studied IO nanoparticles to be bactericidal. The main mechanism by which anti-bacterial drugs and antibiotics work is
via oxidative stress generated by reactive oxygen species (ROS) [299]. ROS, including superoxide radicals ($O_2^-$), hydroxyl radicals (-OH), hydrogen peroxide ($H_2O_2$), and singlet oxygen ($^1O_2$), can cause damage to proteins and DNA in bacteria [300]. Park et al. also demonstrated anti-bacterial activity from silver metals due to ROS generation [301]. In this case, metal oxide $Fe_3O_4$ particles could be the source that created ROS leading to the inhibition of $S. aureus$. A similar process was described by Keenan et al. in which $Fe^{2+}$ reacted with oxygen to create hydrogen peroxide [302]. This $H_2O_2$ consequently reacted with ferrous irons via the Fenton reaction and produced hydroxyl radicals which are known to damage biological macromolecules [288].

Other research has demonstrated that the small size of nanoparticles can also contribute to their bactericidal effects. For example, Lee et al. reported that the inactivation of $Escherichia coli$ by zero-valent iron nanoparticles [303] could be due to the penetration of small particles (sizes ranging from 10-80 nm) into $E. coli$ membranes. Nano- $Fe^0$ could then react with intracellular oxygen, leading to oxidative stress and eventually cause disruption of the cell membrane. Several other studies on ZnO and MgO nanoparticles also concluded that antibacterial activity increased with decreasing particle size [304-306]. Our studies on the uptake of citric acid coated $Fe_3O_4$ nanoparticles into $S. aureus$ and $Pseudomonas aeruginosa$ also showed that the nanoparticles with sizes around 10 nm can penetrated into bacteria (Figure 5.8). The nanoparticles were stabilized with citric acid similarly to the nanoparticles used in chapter 2. Unlike osteoblasts, bacteria have no endocytosis mechanism. The internalizing pathways of nanoparticles into bacteria are still under investigation.
Figure 5.8 *Staphylococcus aureus* (a) and *Pseudomonas aeruginosa* (b) in the presence of citric acid coated Fe$_3$O$_4$ nanoparticles. Images from TEM. Scale bars = 100nm. The arrows demonstrate nanoparticles inside bacteria.

Another mechanism of the bactericidal activity of nanoparticles is intracellular thiol depletion. Thiol groups are very important for the overall health of cells. Intracellular thiols protect the cells from damage caused by free radicals, toxins, carcinogens, etc. A previous study by Tran et al. on selenium nanoparticles showed that in the presence of nanoparticles, thiol levels in *S. aureus* were greatly decreased. Consequently, bacteria cell counts were less in the presence of selenium nanoparticles compared to the control samples without any nanoparticles [307].

In this study, the concentration of nanoparticles had a significant contribution on *S. aureus* inhibition. A similar concentration-dependent relationship was observed by Kim et al. [308] and Zhang et al. [304] when they investigated the antimicrobial effects of Ag and ZnO nanoparticles on *S. aureus* and *Escherichia coli* (the highest concentration studied was 0.2 nM and 0.25 g/L respectively). In a study on the bactericidal effect of
iron oxide nanoparticles on *Staphylococcus epidermidis*, Taylor et al. also reported a concentration dependent of bacteria inhibition [309]. Briefly, *Staphylococcus epidermidis* density progressively decreased at time points 12, 24 and 48 hours when incubated with 100 µg/mL, 1 mg/mL, and 2 mg/mL. These concentrations were very close to the effective dose in the current study (3 mg/mL).

In the current study, the live/dead ratio decreased 20% after 24 hours of culture in the presence of IO/PVA nanoparticles. However, in a clinical setting, a complete destruction of bacteria is more preferable. To improve bactericidal activity of magnetic nanoparticles, the particles can be functionalized with other metal particles or compounds that showed stronger antibacterial properties (such as Ag, Se, and antibiotics [304], [308]). Using an external magnetic field, such nanoparticle systems can be directed to penetrate and destroy biofilm.

It is also important to note that IO nanoparticles do not negatively influence all cells. Specifically, osteoblast (bone forming cell) proliferation was enhanced in the presence of Fe₂O₃ nanoparticles (at 4.25 mg/mL, the same order of magnitude as the effective bacteria inhibition concentration found in this study) [79]. Such results showed that IO nanoparticles could have a dual therapeutic function which can enhance bone growth and inhibit bacterial infection.

5.4 Conclusions

Stable IO/PVA nanoparticles were successfully synthesized in this chapter. The particles were characterized with TEM, dynamic light scattering, XRD and VSM. A live/dead assay showed that at the highest dose of iron oxide nanoparticle (3 mg/mL), the
growth of *S. aureus* was inhibited significantly compared to the control samples. Further studies on the role of iron oxide nanoparticles on the generation of ROS and depletion of intracellular thiol level can clarify the antibacterial mechanisms. More studies also should investigate the bactericidal effect of IO nanoparticles on other types of bacteria (such as antibiotic resistance *Staph. aureus* or *Pseudomonas aeruginosa*) for potentially widening such anti-bacterial applications.
CHAPTER 6. CONTROL OF MAGNETIC NANOPARTICLES USING AN EXTERNAL MAGNETIC FIELD FOR DRUG DELIVERY APPLICATIONS

In the previous chapters, magnetic nanoparticles were synthesized, modified and characterized using various methods (such as XRD, TEM, XPS, FTIR, VSM, etc). The magnetic nanoparticles proved to be a potential treatment for osteoporosis (increasing bone growth) and decreasing bacterial infections. In particular, when osteoblasts were cultured in the presence of 100 $\mu$g/mL HA coated Fe$_3$O$_4$ nanoparticles for 5 days, cell density increased compared to control samples (no particles). After 21 days of culture in the presence of 200 $\mu$g/mL HA coated Fe$_3$O$_4$ nanoparticles, osteoblast long term markers such as alkaline phosphatase synthesis, collagen synthesis and calcium deposition were also improved compared to the controls. However, the applicability of magnetic nanoparticles as a drug delivery system for these applications involves the ability to control the location of the nanoparticles using external magnetic fields. In this chapter of the thesis, focus will be placed on evaluating magnetic fields and magnetic forces from various settings of a magnetic coil and also permanent magnets to control magnetic nanoparticle placement in the body.

6.1 Theory

6.1.1 Magnetic force on a magnetic nanoparticle

To understand the magnetic force on a magnetic nanoparticle, we start with the assumption that an external magnetic field acts on nanoparticles the same way as a magnetic dipole [310]. The magnetic force is given by:

$$ \mathbf{F}_m = (\mathbf{m} \cdot \nabla) \mathbf{B} $$  \hspace{1cm} (6.1)
where \( m \) is magnetic dipole moment and \( B \) is the magnetic induction due to the external field. Equation 6.1 suggests that the force on and the movement of magnetic nanoparticles are governed by the gradient of the magnetic field rather than the magnetic field itself.

For a magnetic nanoparticle, \( m \) can be written as \( m = V_p M_p \), where \( V_p \) is the volume of the particle and \( M_p \) is the magnetization of the particle. Magnetization, \( M_p \), is related to the external field strength \( H \) by the formula: \( M_p = \Delta \chi H \), where \( \Delta \chi = \chi_p - \chi_w \) is the effective susceptibility of the particle relative to the water (\( \chi_p \) and \( \chi_w \) are the susceptibility of the particle and of water, respectively. For this analysis, we assume that the magnetic susceptibility of water is essentially that of free space. However, it is important to note that the susceptibility of the surrounding media can have effects on the magnetic force exerted on the nanoparticles [311-312]. Since the nanoparticle is superparamagnetic, we assume that its magnetization is saturated at all values of an external magnetic field, which means that: \( \Delta \chi = \chi_p = \frac{M_{sp}}{H} \), where \( M_{sp} \) is the saturation magnetization of the nanoparticle. For the case of a dilute suspension of nanoparticles in water, we can utilize the relationship \( B = \mu_o H \), where \( \mu_o \) is the permeability of free space. Thus, equation 6.1 becomes:

\[
F_m = V_p \frac{M_{sp}}{B} (B \cdot \nabla)B
\]  

(6.2)

Therefore, to understand the magnetic force acting on a nanoparticle, it is essential to establish the value of the external magnetic field.
For that purpose, the magnetic fields due to magnetic coil and permanent magnets will be analyzed. Based on the data of the magnetic fields and field gradients, the magnetic forces acting on a nanoparticle will be calculated. Finally, the calculated magnetic fields and forces will be plotted against x and z using Matlab.

6.1.2 Magnetic field and force of a coil

First, we consider a coil with N turns and radius R that carries a current I (Figure 6.1). A magnetic nanoparticle with volume $V_p$ and saturation magnetization $M_{sp}$ is located on the z axis at distance $z$ from the origin.

![Figure 6.1 A magnetic coil with radius R and N turns.](image)

Due to symmetry, on the z axis, $B = B_z$. Equation 6.2 becomes:

$$F_m = V_p M_{sp} \frac{dB_z}{dz} \tag{6.3}$$

The magnetic field on the z axis is given by:
\[ B_z = \frac{\mu_o}{4\pi} \frac{2\pi R^2 I N}{(z^2 + R^2)^{3/2}} \]  

(6.4)

Therefore,

\[ \frac{\partial B_z}{\partial z} = -\frac{3}{2} \frac{\mu_o R^2 I N}{(z^2 + R^2)^{5/2}} \frac{z}{(z^2 + R^2)^{5/2}} \]  

(6.5)

Hence, the force acting on a nanoparticle at (0, 0, z) is:

\[ F_m = -\frac{3}{2} \mu_o V_p M_s R^2 I N \frac{z}{(z^2 + R^2)^{5/2}} \]  

(6.6)

6.1.3 Magnetic field and force of permanent bar magnets

Next, we analyze the magnetic field and the force due to permanent magnets. The case of a bar permanent magnet with a magnetization \( M_s \) along the z axis was calculated in ref [313]. Specifically, the width of the magnet is 2w and the thickness is 2h (Figure 6.2).
Following ref [313], the x-component of the magnetic field from a permanent magnet is written as:

\[
B_x(x, y, z) = \frac{\mu_0 M_s}{4\pi} \sum_{k=1}^{2} \sum_{m=1}^{2} (-1)^{k+m} \ln \left[ F(x, y, z, x_m, y_1, y_2, z_k) \right]
\]

where \((y_1, y_2)=(-w, w), (x_1, x_2)=(-w, w)\) and \((z_1, z_2)=(-h, h)\) and

\[
F(x, y, z, x_m, y_1, y_2, z_k) = \frac{(y - y_1) + [(x - x_m)^2 + (y - y_1)^2 + (z - z_k)^2]^{1/2}}{(y - y_2) + [(x - x_m)^2 + (y - y_2)^2 + (z - z_k)^2]^{1/2}}
\]
The y-component is similar to the x-component with the only modification as the exchange of x and y variables as:

\[ B_y(x, y, z) = \frac{\mu_0 M_s}{4\pi} \sum_{k=1}^{2} \sum_{m=1}^{2} (-1)^{k+m} \ln \left[ H(x, y, z, x_m, y_1, y_2, z_k) \right], \quad (6.9) \]

where

\[ H(x, y, z, x_m, y_1, y_2, z_k) = \frac{(x-x_1)+(y-y_1)^2+(z-z_k)^2}{(x-x_2)+(y-y_1)^2+(z-z_k)^2} \]

The z-component is given by:

\[ B_z(x, y, z) = \frac{\mu_0 M_s}{4\pi} \sum_{k=1}^{2} \sum_{n=1}^{2} \sum_{m=1}^{2} (-1)^{k+n+m} \times \tan^{-1} \left[ \frac{(x-x_n)(y-y_m)}{(z-z_k)} \times g(x, y, z, x_n, y_m, z_k) \right], \quad (6.11) \]

where

\[ g(x, y, z, x_n, y_m, z_k) = \frac{1}{[(x-x_n)^2+(y-y_m)^2+(z-z_k)^2]^{1/2}} \quad (6.12) \]

From equations (6.7), (6.9) and (6.11), the magnetic field generated by the permanent magnet can be determined at any point outside the magnet.

Since the magnetic force depends on the gradient of the magnetic field, it is logical to combine two different permanent magnets to create greater force. For example, assume that we have two identical magnets with dimensions and magnetization the same as the above case and two magnets are placed next to each other with magnetization pointing in opposite directions (Figure 6.3). The Cartesian coordinate is placed at the center of the system. First, the magnetic field due to the system of two magnets is
calculated. Second, the gradient of the field is calculated on lines parallel to the x axis (three lines (x, 0, z₀) are shown in Figure 6.3 with z₀ = 2, 1, 0.4). In practical applications, these lines can be interpreted as a microvessel on the bone surface, and the magnets are placed there to draw nanoparticles from the microvessel into bone. The field and field gradient along the z axis is also calculated. Finally, the force is calculated using the information of a magnetic field and field gradient. Due to the symmetry of the problem, component Bₐ is always equal to 0 on all lines that are calculated and, thus, is not considered.

![Figure 6.3](image)

Figure 6.3 System of two permanent magnets placed next to each other.

When two magnets are placed next to each other, the total magnetic field of the two magnets is the superposition of the magnetic fields due to each magnet. Similar to equation 6.7, the component Bₓ is given by:
\[ B_x(x, z) = \frac{\mu_0 M_s}{4\pi} \left\{ \ln \frac{(x + 2w)^2 + (z - h)^2}{(x + 2w)^2 + (z + h)^2} + \ln \frac{(x - 2w)^2 + (z - h)^2}{(x - 2w)^2 + (z + h)^2} - 2 \right. \\
\times \ln \frac{x^2 + (z - h)^2}{x^2 + (z + h)^2} \right\} \\
(6.13) \]

and the z-component is given by:

\[ B_z(x, z) = \frac{\mu_0 M_s}{2\pi} \left\{ \tan^{-1} \frac{2h(x + 2w)}{(x + 2w)^2 + z^2 - h^2} + \tan^{-1} \frac{2h(x - 2w)}{(x - 2w)^2 + z^2 - h^2} - 2 \right. \\
\times \tan^{-1} \frac{2hx}{x^2 + z^2 - h^2} \right\} \\
(6.14) \]

Therefore, the gradient of the field can be written as:

\[ \frac{\partial B_x}{\partial x} = \frac{2\mu_0 M_s z h}{\pi} \left\{ \frac{(x + 2w)}{[(x + 2w)^2 + (z + h)^2][(x + 2w)^2 + (z - h)^2]} \\
+ \frac{(x - 2w)}{[(x - 2w)^2 + (z + h)^2][(x - 2w)^2 + (z - h)^2]} - 2 \right. \\
\times \frac{x}{[x^2 + (z + h)^2][x^2 + (z - h)^2]} \right\} \\
(6.15) \]
\[
\frac{\partial B_x}{\partial z} = \frac{\mu_0 M_s h}{\pi} \left\{ \frac{z^2 - h^2 - (x + 2w)^2}{[(x + 2w)^2 + (z + h)^2][(x + 2w)^2 + (z - h)^2]} \right.
\]
\[
+ \frac{z^2 - h^2 - (x - 2w)^2}{[(x - 2w)^2 + (z + h)^2][(x - 2w)^2 + (z - h)^2]} - 2
\]
\[
\times \frac{z^2 - h^2 - x^2}{[x^2 + (z + h)^2][x^2 + (z - h)^2]} \right\}
\]  
(6.16)

\[
\frac{\partial B_y}{\partial x} = \frac{\mu_0 M_s h}{\pi} \left\{ \frac{z^2 - h^2 - (x + 2w)^2}{4h^2(x + 2w)^2 + [(x + 2w)^2 + z^2 - h^2]^2} \right.
\]
\[
+ \frac{z^2 - h^2 - (x - 2w)^2}{4h^2(x - 2w)^2 + [(x - 2w)^2 + z^2 - h^2]^2} - 2
\]
\[
\times \frac{z^2 - h^2 - x^2}{4h^2x^2 + [x^2 + z^2 - h^2]^2} \right\}
\]  
(6.17)

\[
\frac{\partial B_z}{\partial z} = \frac{2\mu_0 M_s h}{\pi} \left\{ \frac{-(x + 2w)}{4h^2(x + 2w)^2 + [(x + 2w)^2 + z^2 - h^2]^2} \right.
\]
\[
+ \frac{-(x - 2w)}{4h^2(x - 2w)^2 + [(x - 2w)^2 + z^2 - h^2]^2} - 2
\]
\[
\times \frac{x}{4h^2x^2 + [x^2 + z^2 - h^2]^2} \right\}
\]  
(6.18)

From equations 6.13 - 6.18, magnetic force on a magnetic nanoparticle on previously stated lines \((x, 0, z_0)\) can be calculated:

\[
F_x = V_p \frac{M_s p}{B} \left\{ B_x(x, z) \frac{\partial B_x(x, z)}{\partial x} + B_z(x, z) \frac{\partial B_x(x, z)}{\partial z} \right\}
\]  
(6.19)
and

\[ F_z = V_p \frac{M_{sp}}{B} \left\{ B_x(x, z) \frac{\partial B_z(x, z)}{\partial x} + B_z(x, z) \frac{\partial B_z(x, z)}{\partial z} \right\} \]  \hspace{1cm} (6.20)

where \( B \) is the magnitude of magnetic induction: \( B = (B_x^2 + B_z^2)^{1/2} \).

### 6.2 Results and discussion

For all analyses in this current chapter, we assumed that the magnetic susceptibility of media (water) was that of free space. The nanoparticle was Fe₃O₄ with a radius 10 nm, and had a density \( \rho_p = 5000 \text{ kg/m}^3 \), and a magnetization \( M_{sp} = 4.78 \times 10^5 \text{ A/m} \) [314]. Thus, the volume of the nanoparticle was \( V_p = 4.18 \times 10^{-24} \text{ m}^3 \).

#### 6.2.1 Magnetic field and force of a coil

We used equation 6.6 to study the magnetic field of a coil and assume that the coil was made of 150 turns of copper wire with a diameter of 0.405 mm (gauge 26). The diameter of the coil was 20 cm and the electric source for the coil was 20V DC. From these data, the coil resistance was calculated to be \( R = 12.3 \Omega \) and the current \( I = 1.63 \text{ A} \).

The magnetic field amplitude on the z axis was calculated and plotted with respect to z (Figure 6.4). The field reaches a maximum value of \( B_z = 1.55 \times 10^{-3} \text{ T} \) at the center of the coil and decreased gradually as z increased. At \( z = 20 \text{ cm} \), the magnetic field strength decreased 10 times to around \( 1.4 \times 10^{-4} \text{ T} \).
Using equation 6.5, the gradient of the field was calculated and plotted with respect to $z$ (Figure 6.5). The gradient at the center of the coil was 0. It reached a maximum value of $\frac{\partial B_z}{\partial z} = -0.0132$ T/m at $z = 5$ cm. The field gradient decreased as $z$ increased but was always negative. The negative sign of the field gradient and the force indicated that the particle was attracted toward center of the coil.
Therefore, the maximum magnetic force on nanoparticles is:

\[
F_m = V_p M_s \frac{\partial B_z}{\partial z} = 4.18 \times 10^{-24} \text{(m}^3\text{)} \times 4.78 \times 10^5 \left(\frac{A}{m}\right) \times 0.0132 \left(\frac{T}{m}\right) = 2.6 \times 10^{-20} \text{N} = 2.6 \times 10^{-8} \text{pN}
\]

6.2.2 Magnetic field and force of permanent bar magnets

For the analysis of permanent magnets, we used equation 6.12 to calculate magnetic field distribution on a surface parallel to the (x,y) surface at z = 0.4, 1 and 2 cm. The magnet chosen was a rare-earth NdFeB magnet with a magnetization of \(M_s=1\times10^6\) A/m [315]. The magnet dimensions were 2.4 × 2.4 × 0.4 cm (position of the edge was (-1.2,1.2); (-1.2,1.2); (-0.2,0.2)). Plots of \(B_z\) at z = 2, 1 and 0.4 cm are shown in Figure 6.6, Figure 6.7, and Figure 6.8. At z = 2 cm and z = 1 cm, \(B_z\) was the highest at the center of
the magnet and reduced gradually to the edge of the magnet. For \( z = 2 \) cm and 1 cm, the highest value of \( B_z \) was 0.032 T and 0.095 T respectively. This was expected as \( B_z \) is higher at the \( z \) closer to the magnet surface. For the case when \( z = 0.4 \) cm, \( B_z \) dips at the center of the magnet but peaks further away near the edge. These results can be explained by the current model [313], in which the magnet is replaced by equivalent current sheets along the edges. At \( z \) closer to the magnet surface, the field is stronger near the current sheets. As \( z \) moves further away from the magnet, the vertical value decreases greatly on the edges and peaks at the center. The highest value of \( B_z \) at \( z = 0.4 \) cm is 0.19 T.

Figure 6.6 Magnetic field \( B_z \) at \( z = 2 \) cm from the center of the magnet.
The magnetic field due to a set of two permanent magnets is the superposition of the magnetic field produced by each magnet. Using equation 6.14, the magnetic field $B_z$ can be calculated in a similar way to the case of only one magnet. Plots of $B_z$ at $z = 2$, 1 and 0.4 cm are shown in Figure 6.9, Figure 6.10, and Figure 6.11.
Figure 6.9 Magnetic field $B_z$ at $z = 2$ cm from the center of two magnets.

Figure 6.10 Magnetic field $B_z$ at $z = 1$ cm from the center of two magnets.
Figure 6.11 Magnetic field $B_z$ at $z = 2$ cm from the center of two magnets.

Due to the superposition of magnetic fields produced by the two magnets, the peaks of $B_z$ were shifted toward the center of the system. Since the magnetizations of two magnets were placed in opposite directions, the magnetic fields of the systems have two peaks at $B_z > 0$ and $B_z < 0$. For $z = 2$ cm and $z = 1$ cm, the highest values of $B_z$ were 0.03 and 0.1 T, respectively. The dips at the center of the magnets were again observed for the case of $z = 0.4$ cm. There was a drastic change in $B_z$ at the edge between the two magnets. It is possible that the highest force can be observed here.

Next, we studied the magnetic field on a line parallel to the x axis and cross the z axis at 0.4, 1 and 2. Using equations 6.13 and 6.14, $B_x(x)$ and $B_z(x)$ at $z = 2$, 1 and 0.4 cm were calculated. $B_x$ and $B_z$ was plotted with respect to x (Figure 6.12, Figure 6.13, and Figure 6.14). At all $z$, $B_x$ was symmetric through the line $x = 0$. $B_x$ peaks at $x = 0$ and had maximum values of 0.017, 0.042 and 0.048 T at $z = 2$, 1, 0.4 cm, respectively. In contrast, $B_z$ was symmetric through the origin and was always equal to 0 at $x = 0$. 

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Figure 6.12 $B_x$ and $B_z$ at $z = 2$ cm as a function of $x$.

Figure 6.13 $B_x$ and $B_z$ at $z = 1$ cm as a functions of $x$. 
Figure 6.14 $B_x$ and $B_z$ at $z = 0.4$ cm as a functions of $x$.

Using numerical derivatives, the magnetic field gradients were obtained from the magnetic field data. Gradients on the lines $(x, 0, z_0)$, where $z = 2, 1, 0.4$ cm, were considered. $\frac{\partial B_x}{\partial x}, \frac{\partial B_z}{\partial x}$ and $\frac{\partial B_x}{\partial z}, \frac{\partial B_z}{\partial z}$ were plotted with respect to $x$ in Figure 6.15, Figure 6.16 and Figure 6.17. It is important to note that $\frac{\partial B_x}{\partial x}$ reached a maximum value at $x = 0$ and as we moved closer to the surface ($z$ decreases), the peak increased drastically from 0.035 T/cm at $z = 2$ cm to 0.17 T/cm at $z = 1$ cm and finally 1.3 T/cm at $z = 0.4$ cm. This gradient can create a larger magnetic force to attract the nanoparticles to the center line between the two magnets.
Figure 6.15 Magnetic field gradients $\frac{\partial B_x}{\partial x}, \frac{\partial B_y}{\partial x}$ and $\frac{\partial B_x}{\partial z}, \frac{\partial B_y}{\partial z}$ with respect to x at z = 2 cm.
Figure 6.16 Magnetic field gradients $\frac{\partial B_x}{\partial x}$, $\frac{\partial B_z}{\partial x}$ and $\frac{\partial B_x}{\partial z}$, $\frac{\partial B_z}{\partial z}$ with respect to $x$ at $z = 1$ cm.
Figure 6.17 Magnetic field gradients $\frac{\partial B_x}{\partial x}$, $\frac{\partial B_z}{\partial x}$ and $\frac{\partial B_x}{\partial z}$, $\frac{\partial B_z}{\partial z}$ with respect to x at z = 0.4 cm.

From the magnetic field and magnetic field gradient data, the force exerted on a nanoparticle on a line parallel to the x-axis at z = 2, 1, 0.4 cm was calculated. $F_x$ and $F_z$ were plotted with respect to x in Figure 6.18, Figure 6.19 and Figure 6.20. As expected, in all cases, $F_x$ was an odd function while $F_z$ was an even function. For our specific application, we are more interested in $F_z$ because it reflects the ability to guide nanoparticles into the bone. For z = 2 cm, $F_z$ peaked at x = ±1.2 cm from the center with
a value of $7.3 \times 10^{-6}$ pN, while for $z = 1$ and 0.4 cm, $F_z$ peaked at $x = 0$ with maximum values of $3.6 \times 10^{-5}$ pN and $2.6 \times 10^{-4}$ pN, respectively. The explanation of this behavior is that near the edge between the two magnets, the field gradient was so high, which resulting in force domination at the edge compared to the center of the magnets. As we moved further away from the magnet, the gradient at $x = 0$ decreased more rapidly compared to the gradient at the center of each magnet. Therefore at $z = 2$ cm, the force due to gradient at the center of each magnet was already greater that the force due to the gradient at $x = 0$.

Figure 6.18 Magnetic force $F_x$ and $F_z$ at $z = 2$ cm as a function $x$. 

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Figure 6.19 Magnetic force $F_x$ and $F_z$ at $z = 1$ cm as a function $x$.

Figure 6.20 Magnetic force $F_x$ and $F_z$ at $z = 0.4$ cm as a function $x$.

To understand more concerning the behavior of $F_z$, the magnetic force along the $z$ axis was calculated. On the $z$ axis, $B_z = 0$, therefore, $B_x = B$. Equation 6.20 becomes:

$$F_z = V_p M_{sp} \frac{\partial B_g(x,z)}{\partial x}$$

(6.21)
A plot of $\log(F_z)$ with respect to $z$ is shown in Figure 6.21. The magnetic force along the $z$ axis decreased very quickly as $z$ increased. Although the magnetic force on the surface of the magnet can reach as high as $7 \times 10^{-3}$ pN, the force decreased 100 fold at $z = 1$ cm and almost 1000 fold at $z = 2$ cm.

![Figure 6.21 Magnetic force $F_z$ along the z axis as a function of z.](image)

The current results suggest that the magnetic force in this magnet system was greatly contained in a very small region near the edge between the two magnets. If the nanoparticles can be placed sufficiently close to the magnet, they are eventually focused at that edge. Compared to the maximum force generated by the coil, the permanent magnet can create a much stronger force (10,000 times higher). This force is also much larger than other factors such as gravitational force ($21 \times 10^{-8}$ pN) or buoyancy ($4.2 \times 10^{-8}$ pN). However, the range of the magnetic force in this case is still very small and thus, limiting the applicability of using magnetic nanoparticles as a drug delivery system. There are several ways to improve the control of nanoparticles using an external
magnetic field. First, a stronger magnet should be used. Second, since the magnetic force depends on the volume of the nanoparticle, increasing particle size can result in higher magnetic force. In this current study, the radius of each nanoparticle was 10 nm. By increasing the nanoparticle radius to 100 nm, the magnetic force can be enhanced 1000 times. However, it is very important to note that increasing particle size can compromise bioactivity aspects of the system. For example, 100 nm radius nanoparticles have less circulation time, are recognized by reticuloendothelial system and, thus, are cleared before be able to react with bone cells [111]. Furthermore, if the nanoparticle is size larger than 25 nm, the particle is no longer superparamagnetic. This can lead to particle agglomeration and also reduce circulation time.

6.3 Conclusions

This chapter of the thesis focused on the ability to control nanoparticles using an external magnetic field. Field analysis was completed for a magnetic coil, a single square permanent magnet and also a system of two permanent magnets. From the magnetic field and field gradient analysis, the magnetic forces were obtained. The results showed that the system of two magnets can focus nanoparticles to the edge between the magnets. The highest force generated was in the range of $7 \times 10^{-3}$ pN. Although this force was large enough for nanoparticle focusing, the active distant was only a few centimeters from the magnet surface. Thus, this external field is more suitable for delivery of nanoparticles to shallow tumors. For osteoporosis applications, the two magnet system showed limitations because the nanoparticles must be controllable from at least 10 cm to 15 cm away. That is the average thigh radius of an adult patient. In a patient suffering from obesity, the task is
even harder. Therefore, generating a high enough magnetic force that is able to focus nanoparticles is a remaining challenge in magnetic based drug delivery system.
CHAPTER 7. SUMMARY

This thesis presented a comprehensive study of a promising magnetic nanoparticle based drug delivery system for osteoporosis treatment and also for fighting bacterial infection. Specifically, in this study, Fe$_3$O$_4$ magnetic nanoparticles were synthesized using a wet chemistry method with a citric acid stabilizer. For osteoporosis treatment, Fe$_3$O$_4$ nanoparticles were coated with hydroxyapatite (HA), the main inorganic biocompatible compound of natural bone. The nanoparticles were characterized using TEM, DLS, XRD, XPS and VSM. The magnetic nanoparticles showed excellent magnetic properties and were superparamagnetic. The magnetic properties were retained after HA coating.

Osteoblast proliferation and differentiation were studied in the presence of uncoated and HA coated Fe$_3$O$_4$ nanoparticles. The results showed that HA coated Fe$_3$O$_4$ nanoparticles were magnetic and enhanced osteoblast density after 5 days of culture (nanoparticle concentration: 100 µg/mL). Most importantly, using these nanoparticles at concentrations of 200 µg/mL improved osteoblast differentiation after 21 days of culture, as shown by greater alkaline phosphatase (ALP) activity, collagen synthesis and calcium deposition compared to control samples without any nanoparticles. These results highlight the potential of using HA coated magnetic nanoparticles to increased bone growth at desirable bone defects sites which should be further studied.

To further understand the mechanism of how the nanoparticles interact and enhance bone cell function, protein adsorption onto the nanoparticle surface was investigated. It was shown that proteins in serum adsorbed on and changed the
Fourier transform infrared (FTIR) spectra of the nanoparticles incubated in serum showed exclusive dips at 2964, 2933 and 2879 cm\(^{-1}\) compared to that of the original nanoparticle. Fibronectin ELISA results showed higher total amounts of fibronectin adsorbed on 200 µg/mL HA coated Fe\(_3\)O\(_4\), which led to better bone cell proliferation and differentiation. It was believed that the increased adsorption of adhesive protein (such as fibronectin and vitronectin) was favorable for osteoblast function. In this study, RGD peptide in fibronectin adsorbed on the nanoparticle surface might have bound to \(\alpha_5\beta_1\), \(\alpha_{IIb}\beta_3\) or \(\alpha_V\beta_3\) integrins on osteblast surfaces. The signals transduced by integrins then promote gene expression and differentiation of osteoblasts.

The molecular mechanisms of enhanced bone cell function were also studied. At the particle concentrations of interest (200, 100 and 12.5 µg/mL), no genotoxicity was detected on genes osteocalcin, type I collagen and cbfa-1. A longer time period of investigation should be completed in the future to signify the difference in gene expression in the presence of these nanoparticles, which may provide further evidence of the molecular recognition of HA coated Fe\(_3\)O\(_4\) nanoparticles by osteoblasts.

The thesis also addressed the nanoparticle uptake route via receptor – mediated endocytosis. A time and concentration dependent endocytosis pathway was observed. A model was developed to study the uptake mechanism. The model predicted the saturation uptake trend in all nanoparticles. From the relationship among the endocytosis rates, the model also predicted the behavior of the uptake curves which resembled the experimental uptake of Fe\(_3\)O\(_4\) and HA coated Fe\(_3\)O\(_4\) nanoparticles. However, further experimental data are needed for the model to be able to mimic the current system. Parameters such as the
number of ligands on the nanoparticle surface, the number of receptors on cell membrane, the exocytosis rate and the recycling rate are priorities in future studies.

The study also investigated the potential of using magnetic nanoparticles as antibacterial agents that can penetrate through a biofilm. Fe$_3$O$_4$ nanoparticles were synthesized in the presence of polyvinyl alcohol (PVA) and characterized using XRD, TEM, VSM and DLS. A live/dead assay showed that at the highest dose of iron oxide nanoparticles (3 mg/mL), the growth of \textit{Staphylococcus aureus} was inhibited significantly compared to the control samples (no nanoparticles). Further studies on the role of iron oxide nanoparticles on the generation of ROS and depletion of intracellular thiol level can clarify the antibacterial mechanisms. More studies also should investigate the bactericidal effect of IO nanoparticles on other types of bacteria (such as \textit{Pseudomonas aeruginosa}), especially the antibiotic resistant ones, for potentially widening such anti-bacterial applications.

Lastly, the thesis evaluated the efficacy of this drug delivery system by calculating magnetic forces on the nanoparticles. The calculation results showed that the forces exerted on nanoparticles due to magnetic coil and permanent magnets in this study were quite small ($2.6 \times 10^{-8}$ pN and $2.6 \times 10^{-4}$ pN at 0.4 cm from the magnet center, respectively). Finding other strategies to increase the ability to control and focus the nanoparticle using external magnets are crucial to ensure the applicability of this drug delivery system.

In conclusion, the magnetic nanoparticle and coating systems developed in this thesis showed great potential as treatments for osteoporosis and bacterial infections. The
application ranges of these nanoparticles are certainly not limited in the aforementioned areas. In the future, using a suitable functionalization technique, a magnetic nanoparticle can be turned into a multifunctional system, a “magic bullet”, which can kill cancer cells, inhibit bacteria, promote bone cells and also provide visualization via MRI and PET scans.
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