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UNIVERSITY OF CALIFORNIA
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Magnetic Nanocomposites and Fields for Tissue Engineering Applications

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Bioengineering

by

Naiyin Zhang

December 2017

Dissertation Committee:

Dr. Huinan Liu, Chairperson
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Dr. Nissim Amos

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The Dissertation of Naiyin Zhang is approved:

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Acknowledgments

I would like to thank the entire dissertation committee: Dr. Huinan Liu, Dr. William Grover, Dr. Yadong Yin and Dr. Nissim Amos for their kind support and encouragement on my project. I would also like to thank all my previous and current lab mates for their generous help. I would like to thank Amy Sallee for her mentoring on cell culture techniques, I would like to thank Ian Johnson, Aaron Cipriano, Qiaomu Tian, Cheyann Wetteland, Wensen Jiang, Nhu Nguyen and Chaoxing Zhang for their advise on my project and kind help with lab techniques and instruments. I would like to thank my undergraduates Andro Azer, Jessica Si'i, Michael Segura, Jonathan Zhang and David Wang for their dedicated work on my sub-projects.

I would like to thank all the staff in Bioengineering department for their professional work and help. I would like to thank Nancy Ford, Hong Xu, Reneisha Wilkes, Isidra Villalobos Martinez, Annette Meneses, Valorie Piper and Sonia Godinez.

I would like to thank people from other programs and univierties for their generous help. I would like to thank Dr. Lawrence D. Longo at Loma Linda University for providing sheep stifle joints for Type II collagen extraction. I would like to thank Dr. Gary Botimer at Loma Linda University for the kind gift of Arithrex Osteochondral Autograft Transfer System (OATS) for harvesting the subchondral bone plugs. I would like to thank Mohammed Aldosary and Dr. Jin Shi for their kind help with hysteresis loop test. I would like to thank Central Facility for Advanced Microscopy (CFAMM) at the University of Cal-

ifornia, Riverside, for the use of SEM, and Ilkeun Lee and Dr. Krassimir Bozhilov for the training on SEM, Mathias Rommelfanger for the training on Sputter Coating. I would like to thank Dr. Perry Cheung from MSE program for his kind training on the use of Instron for mechanical testing.

I would like to thank funding resources Hellman Faculty Fellowship (H.L.) and the University of California Regents Faculty Fellowship (H.L.) for financial support. I would like to thank the Enhancing STEM Transfer and Success through the STEM Pathways Program grant (#P031C110131) for the support of undergraduate student researcher Jessica Si'i. Undergraduate Education Sophomore STEM Success Award for the support of undergraduate student researcher Jonathan Zhang. I would like to thank Stem Cell Center Travel Award and Graduate Student Association Travel Award for supporting me attend 2016 Annual Meeting of Biomedical Engineering Society.

The text of this dissertation Chapter 3, in full, is a reprint of the material as it published in the following journal: ACS Applied Materials & Interfaces, 2015.

Title: Magnetic Nanocomposite Hydrogel for Potential Cartilage Tissue Engineering: Synthesis, Characterization, and Cytocompatibility with Bone Marrow Derived Mesenchymal Stem Cells

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The rest chapters of this dissertation, in part of in full, will be presented in future publications. I would like to thank all the co-authors for their contributions.

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Chapter 4: Adhesion of Bone Marrow-Derived Mesenchymal Stem Cells on Magnetic Poly(glycerol sebacate) Nanocomposites

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Chapter 5: Improved Adhesion of Bone Marrow-Derived Mesenchymal Stem Cells on 3D Porous Magnetic Poly(glycerol sebacate) Nanocomposites

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Chapter 6: The Comparison of Two Static Magnetic Fields on Bone Marrow-Derived Mesenchymal Stem Cell Proliferation and Morphology

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Chapter 7: Influence of A Moderate Electromagnetic Field on Bone Marrow-Derived Mesenchymal Stem Cell Proliferation and Differentiation Behaviors

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Chapter 8: Porous Magnetic Poly(glycerol sebacate) Nanocomposites Increased the Differentiation Potential of Bone Marrow-Derived Mesenchymal Stem Cells

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To my lovely family members for all the support. My beloved grandma Duan Zhang, my grandpa Laibin Xu, my mom Qingzhi Xu, my dad Wenyuan Zhang and my soulmate Junchao Wang.

ABSTRACT OF THE DISSERTATION

Magnetic Nanocomposites and Fields for Tissue Engineering Applications

by

Naiyin Zhang

Doctor of Philosophy, Graduate Program in Bioengineering

University of California, Riverside, December 2017

Dr. Huinan Liu, Chairperson

Current stem cell research often relies on the use of growth factors to stimulate cell proliferation and differentiation for tissue engineering purposes. However, these growth factors are not only short-lived proteins but also expensive resources. Research in biodegradable magnetic nanocomposites is a promising rising field for biomedical applications due to the magnetic response properties of the materials that could provide external physical stimulations as an alternative. This project seeks to investigate the application potential of biodegradable magnetic nanocomposites for bone and cartilage tissue regeneration. Different weight percent of polyvinyl alcohol (PVA) was used to modify the surface of superparamagnetic nanoparticles to reduce aggregation and increase dispersibility in polymer solutions for the synthesis of magnetic nanocomposites. The results showed that 30 wt% of PVA coating was able to provide the best dispersibility compared to all other

groups. Hydrogel-based magnetic nanocomposites were synthesized and the cytocompatibility of magnetic nanocomposite hydrogel with bone marrow-derived mesenchymal stem cells (BMSCs) was higher than pure hydrogel. However, both the hydrogel and magnetic nanocomposite hydrogel completely lost structural integrity within 24 hours of culture with BMSCs, which made it difficult for *in vivo* cell delivery. Another polymer, poly(glycerol sebacate) (PGS) was studied due to its reported elastomeric property. Initial study showed that magnetic PGS nanocomposites without any surface features had low cell adherence. Thus porous structures were created in magnetic PGS nanocomposites to increase cell adhesion. Different weight percent of magnetic nanoparticles (MNPs)-incorporated PGS nanocomposites were synthesized to investigate the effect of MNPs concentration on BMSC proliferation and differentiation behaviors with and without exposure to external electromagnetic field (EMF). The 3-week study showed that BMSCs cultured in magnetic PGS nanocomposite groups had slightly lower cell density but increased ALP activity, calcium deposition, protein and collagen secretion, indicating positive induction towards osteogenesis. This project presented promising porous magnetic PGS nanocomposite scaffolds for bone and cartilage tissue engineering applications.

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Chapter 1

Introduction

1.1 Bone turnover and osteoporosis

In adults, bones are remodeled and rebuilt through a process called bone turnover. It results in the removal of old bones and the replacement by new bones thus ensures the repair of any micro-damages and enables the regulation of mineral homeostasis [1]. When the bone is quiescent, lining cells rest on the surface of bone, while when the bone is activated by mechanical or biochemical stimuli, lining cells retract and the underlying membrane is removed by matrix metalloproteinase. Subsequently, osteoclasts become activated and digest underlying bone, then osteoblasts move into the resorption cavity and form new osteoid. Finally the osteoid becomes calcified and mineralized and form new bones [2, 3]. This process is seen in healthy bone and bone formation keeps in pace with bone destruction. When bone destruction is greater or faster than bone formation, osteoporosis will happen.

Osteoporosis, or porous bone, is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased risk of

fracture of the hip, spine and wrist (Osteoporosis Overview. National Institute of Health, 2012). Bones experiencing osteoporosis have less bone matrix as compared with healthy bone. Older people, especially postmenopausal women, tend to suffer more from osteoporosis. The National Osteoporosis Foundation (NOF) and National Institutes of Health (NIH) reported that by 2015, there were 54 million Americans have osteoporosis and low bone mass and osteoporosis is responsible for two million broken bones every year. \$19 billion dollars are spent each year towards the treatment of broken bones as a result of osteoporosis (The Osteoporosis Report, NOF, 2013; The surgeon general's report on bone health and osteoporosis, 2012). By 2025, experts predict that approximately one in two women and up to one in four men age 50 and older will have broken bones due to osteoporosis and osteoporosis will be responsible for about three million fractures and \$25.3 billion treatment cost annually (What is osteoporosis and what causes it? NOF, 2017).

1.2 Current Treatment Methods of Osteoporosis and Bone Fractures

1.2.1 Nutrition and Exercise

Nutrition and exercise are the necessary components for osteoporosis treatment. The foods we eat everyday should contain a variety of vitamins, minerals and other nutrients that help keep our bodies healthy. Specifically, calcium and vitamin D are the most important nutrients to maintain strong bones. Exercise, on the other hand, is an important approach to prevent and for the treatment of osteoporosis. During activities, stress is put

on bones, which sends a signal to your body that your bones need to be made stronger. New cells are added to strengthen your bones and thus bone mineral density is increased (Osteoporosis Overview, NIH, 2015).

1.2.2 Medication

However, nutrition and exercise are mainly more effective in preventing osteoporosis and as supplemental treatment approach when osteoporosis happens. Current major treatment methods for osteoporosis are drugs. The goal of treatment is the reduction of fractures which can be achieved by decreasing bone resorption and/or by increasing bone formation [4, 5]. The predominant FDA approved drug for osteoporosis therapy is bisphosphonate and a new competitor, denosumab, which can inhibit bone resorption through the inhibition of RANKL activity on the surface of osteoclasts [4]. The only FDA approved anabolic drug that can stimulate bone formation is teriparatide. It is a segment of human parathyroid hormone and can activate osteoblast functions.

Among those drugs, bisphosphonate is the easiest to use. It has been shown to increase bone mineral density and has a terminal half-life of 10 years in bone [5, 6]. However, the long-term treatment will cause osteonecrosis of the jaw and atypical femoral subtrochanteric fracture [4, 5]. Denosumab can reduce fractures efficiently but it can also affect immune system because RANKL is expressed not only on bone cells but also by the immune system [5]. It is expensive compare to other antiresorptive drugs and has a short terminal half-life of only several months [4]. Teriparatide increases bone mineral density

and new bone formation, with a successful rate as high as 60% [7]. However, it also has disadvantages. Not all patients respond to this drug and have a limited 2-year-cycle of treatment.

1.2.3 Stem Cell Therapy

Stem cells have clonogenic and self-renewing capabilities and differentiate into multiple cell lineages [8]. Because stem cell therapy has attracted more and more attention, some scientists have attempted to use stem cells such as bone marrow-derived mesenchymal stem cells (or bone marrow stromal cells [BMSCs]), adipose-derived mesenchymal stem cells and umbilical cord blood-derived stem cells for osteoporosis treatment [6, 7, 9]. Stem cell therapy can also reduce susceptibility of fractures and augment lost mineral density. It has some dominant advantages such as increase or restore the function of resident stem cells when compared to drug treatment [7]. But it also faces many problems for example the uncertainty of stem cell fate and biodistribution following cell transplantation. It has been reported that mesenchymal stem cells do not spontaneously engraft and differentiate upon infusion [7].

While embryonic stem cells (ESC) have the ability of differentiating into different cell types, adult stem cells can only differentiate into limited cell types, but they are not under debate as ESC does. BMSC is a kind of multipotent stem cell that can develop into more than one cell type such as osteoblast, chondrocyte, fat cell, muscle cell, neuron and epithelial cell. BMSCs transplantation is easy to use clinically because cells can be easily

obtained and multiplied without losing their differentiation capacity [10]. Naturally, stem cell differentiation occurs in a specific environment which includes growth factors, inner stimulation, molecular interactions and so on, thus requires a long period for the stem cell to respond and differentiate into the cell types that is needed for bone repair. Thus, if we can apply some exterior stimulation to help induce cell differentiation *in vitro* then transplant them into bone tissue, we can control bone repair more efficiently and effectively.

1.3 Magnetic Nanocomposite Materials as Tissue Engineering Scaffolds

When fracture happens, external fixation device in combination with internal fixation device is used to keep fractured bone stabilized and in alignment, thus ensures the bone in an optimal position during the healing process. The internal fixation implants usually contain plates, screws, nails and wires that are made from stainless steel and titanium, which are clinically proved to be durable and strong, however, a secondary surgery is needed to remove those implants after the fracture is healed. This procedure is usually painful and requires a second recovery process because the implants might have tissue-ingrowth during the healing process and makes the removal-surgery slow and painful.

In this case, tissue engineering provides an approach to heal and regenerate tissue *in situ* or *in vivo* in the hopes of developing alternative treatments for damaged tissues or organs [11]. It provides promising solutions to bone damage such as bone fracture, degenerative diseases, necrosis or tumor. Tissue engineered scaffolds attempt to mimic the extracel-

lular matrix (ECM) in order to provide a suitable environment for cell growth, migration and differentiation. Scaffolds for hard tissues such as bone, require certain mechanical strength, are engineered to meet those requirements [12]. Bioresorbable and biocompatible materials that could support bone recovery during the healing and degrade into biocompatible substances after bone-healing are good candidates for implants to avoid secondary-surgery and reduce pain.

Due to the requirement of mechanical strength, hydroxyapatite (HA) and its composite materials are widely investigated for bone tissue engineering [13]. More recently, polymers such as poly(lactate-co-glycolic acid) (PLGA) [14] and polycaprolactone (PCL) [15] are studied for the repair of small bone defects due to their high fracture toughness when compared with ceramics. However, either ceramic or polymer alone has drawbacks such as lack of response to external stimuli or lack of compressive strength and tensile strength to satisfy the requirement of mechanical properties of bone, respectively [16].

At the same time, magnetic nanoparticles (MNPs) are under intense investigations because of their unique magnetic property when compared with other nanomaterials, which makes them responsive to external magnetic fields (MF) [17] and potential candidate for drug delivery. In addition, they have high specific surface area and potential to induce hyperthermia that is also benefit in other biomedical applications. In 1990s, MNPs were first incorporated into composites, which underwent evaluation of physicochemical properties [18]. However, investigations on the biological properties of magnetic nanocomposites were ignored until recently. Meanwhile, magnetic field (MF) have attracted attention due to

their ability to manipulate MNPs. MFs are proved to have positive effects on bone cell proliferation and differentiation. For example, a study from Jaber et al. showed that a static magnetic field (SMF) could improve the rate of matrix formation and cartilage healing in the right medial femoral condyle of rabbits. Additionally, Tsai et al. found that the production of alkaline phosphatase from human mesenchymal stem cells was significantly enhanced at day 7 under pulsed electromagnetic field (EMF) treatment with extremely low frequency of 7.5 Hz [19, 20]. However, the full range biological effects, both *in vitro* and *in vivo*, remain unclear [21]. More information about MF will be discussed in the next section.

Inspired by the superparamagnetic properties of MNPs with a small diameter (less than 20 nm) and bioeffects of MFs [22], some researchers have studied cell behaviors under the co-influence of magnetic composite scaffolds and MFs. Panseri et al. found that MNPs and MFs have synergistic effects on promoting osteoblast-like cell proliferation, which represents a promising future for bone tissue engineering [23]. In addition to functioning as a support for bone tissue, magnetic composite scaffolds can be used as a drug delivery system and hyperthermia treatment for tumor as well [24, 25]. Through chemical cross-linking or physical interactions, MNPs can adsorb or attach to polymer chains so as to add new properties to the polymers such as responsiveness to mechanical, optical, thermal, magnetic or electric stimuli, etc. [26]. Li et al. claimed that magnetic hydrogel based scaffolds have the ability to stabilize and carry growth factors or other biological agents bound to MNPs through an external MF, providing a nutritious environment for initial cell proliferation [27].

1.4 Biomedical Applications of Magnetic Fields

Magnetic field stimulation has been proved to have influence on stem cell differentiation. The energy exchange in the magnetic field can interact with cell molecules, which changes their natural kinetics or structures and in consequence, induces their function in cell differentiation. These effects depend on the frequency, density and uniformity of the magnetic field, while exposure time, cell type, culture environment and other factors may also have influence on final impacts [28].

1.4.1 Static Magnetic Fields (SMFs)

Here we define different intensity SMF as weak (<1 mT), moderate (1 mT to 1 T) and strong (>1 T).

Weak SMFs

Magnetic field of the Earth is also known as the geomagnetic field. Its magnitude at the surface of the Earth ranges from 25 - 65 μ T and all living organisms are exposed to it. History proved that we are safe in this MF environment, but what about the MF whose magnitude is higher than that?

M. Bekhite et al. showed that cardiomyocyte differentiation of Flk-1⁺ cells derived from mouse embryonic stem cells (ESCs) was increased in a 50 Hz, 1 mT SMF, which was due to the Ca²⁺ influx under the influence of EMF [29]. On the tumor study, J. Hu et al. showed that mice exposed to a weak intensity field (1 - 5 nT) forming smaller tumors than

mice exposed to sham or stronger intensity (2 - 5 μ T) fields, and their immunochemistry analysis of tumors from those mice suggested that those differences may be caused by alterations in leukocyte infiltration or vascularization [30]. The results of those two studies indicated that even the weak intensity of SMF could have influence on cells.

Moderate SMFs

There are lots of studies focused on the influence of moderate SMFs (1 mT to 1 T) on different cells or cell functions. The study combined X-ray with a 15 mT SMF showed that bone marrow stem cells treated with SMF after exposed in X-ray showed a significant increase in the number of cells in G₂/M phase, while cells encountered with SMF alone were not affected, which means SMF could intensify the effects of X-ray [31]. M. Elblbesy showed that a SMF system with intensity from 1 mT to 1 T increased erythrocytes surface area and reduced their sphericity, and they had the tendency to adhere with each other which was not a good phenomenon [32]. There are also studies showed that 7 mT SMF in the presence of Fe²⁺ ions can increase the concentration of oxygen free radicals which may lead to cell death [33]; and mitogenic response was reduced in lymphocytes exposed in a 25 - 150 mT SMF [34]; and an experiment with peripheral blood mononuclear-like cells under a 10 mT SMF showed that the time to induce calcium current in the cells was always less than the situation without magnetic field application [35].

SMF can also have many benefits in studies. F. Jaber et al. showed a 40 mT SMF could improve repair of cartilage damage [19]; the metabolism and respiration of

mitochondria exposed in a 100 mT SMF were all enhanced [36]; the current of Na^+/K^+ pump in the snail nervous system was increased after exposure to a 10 mT SMF, which accompanied with the significant increase in the Na^+/K^+ pump α -subunit expression in the neuronal plasma membrane area [37]; extracellular matrix expression of MG63 cells on the PLLA substrate was promoted when the cells were exposed in a 0.4 T SMF, and the alkaline phosphatase specific activity in the cells was also significantly increased [38]; SMF can also increase biomineralization and osteocalcin secretion rate of osteoblasts [39] and enhance migration of some type of cells [40].

Strong SMFs

The effect of strong SMFs (>1 T) was also investigated by researchers. Rat astrocytes exposed in a 2.1 T SMF did not show significant change in their viability and morphology [41]; Hela cell exposed in a gradient SMF of 6 T with 60 T/m exhibited stream-like cell distribution patterns in the direction of the applied MF gradient, which indicated the possibility of using SMF to guide cell orientation [42]. An interesting study conducted by Y. Eguchi et al. showed that after 8 T, 60 h SMF exposure, Schwann cells alone oriented parallel to the magnetic field; while in the mixture of Schwann cells and bovine skin collagen I, Schwann cells oriented in the direction perpendicular to the MF. From all of the reports mentioned above, we can see different SMFs may have totally adverse effects on cell functions.

1.4.2 Electromagnetic Fields (EMFs)

An electromagnetic field (EMF) is a physical field produced by electrically charged objects. It affects the behavior of charged objects in the vicinity of the field. With different frequency, the EMFs could have diverse effects on living organisms exposed in the field [43].

Low Frequency EMFs

Extremely low frequency (ELF) and low frequency (LF) EMFs are contained in this category, which refers to EMF with radio frequencies less than 300 kHz. Different LF-EMFs on multiple kinds of cells and cell functions were investigated.

S. Kleijn et al. show that 3 μ T, 20 - 5000 Hz ELF-EMF did not modulate the innate immune response of human primary cells after toll-like receptor signaling stimulation in vitro [44]. A 50 Hz and 1 mT EMF was reported to have no effect on keratinocyte cell viability while reduced its gene expression [45]. Those results showed there were insignificant effect on cell functions, while in other cases, the effects were significant.

J. Phillips et al. showed that EMF could cause DNA damage [46]; N. Borhani et al. indicated that the EMF exposure in preimplantation stage could have detrimental effects on female mouse fertility and embryo development [47]; A. Lisi et al. showed a 7 Hz, 100 μ T EMF could promote differentiation in the human epithelial cell line HaCat [48]. LF-EMF could also affect protein tyrosine phosphorylation in culture Chinese hamster lung cells [49] and play a modulating role in hMSC osteogenesis [20], muscle cell redox state [50] and neurite outgrowth [51] etc.

High Frequency EMFs

High frequency electromagnetic field (HF-EMF) refers to the magnetic field with intensity more than 300 kHz. Due to their high magnitude, their effects on cell functions were carefully evaluated.

W. Stankiewicz et al. showed that the immune activity of responding lymphocytes and monocytes can be additionally intensified by 900 MHz microwaves [52], while T. Atay et al. declared a 1800 MHz HF-EMF had no effect on bone tissue with regard to osteoporosis [53], and erythrocyte aggregation was also not effected by 1800 MHz cell phones. Those contrasts indicate that different tissue or cells may have different tolerance to HF-EMF. The study of E. Markova et al. showed that stem cells were more sensitive to microwave exposure and react to more frequencies than do differentiated cells, which may be important for cancer risk assessment [54]. Other studies also showed HF-EMF can regulate gene expression [55], increase protein synthesis [56], decrease neuron latent period [57] and induce pyramidal cell loss in hippocampus [58] and so on.

Similar to SMF and LF-EMF, HF-EMF with different conditions can have diverse influence on cell functions. Some of the effects are disadvantageous which need people to pay attention and find solutions to reduce the risks; while some of the effects are beneficial which can be utilized to biomedical applications.

1.5 Project Description

The previous sections introduced the background of this project and the potential applications of magnetic nanocomposites with external magnetic fields for various biomedical applications. However, there are few studies focused on the coupled effects of magnetic nanocomposite scaffolds and magnetic fields for bone and cartilage regeneration applications.

Thus, the thesis presented here is focused on defining the ability of magnetic nanocomposite scaffolds coupled with magnetic fields to induce BMSCs differentiation for bone and cartilage regeneration. In the following chapters, I will describe the synthesis and cytocompatibility test of magnetic nanocomposite scaffolds, the design and fabrication of external magnetic field generating device and the study of BMSCs proliferation and differentiation behavior when exposed to magnetic nanocomposites and magnetic fields individually and together.

Chapter 2

Polyvinyl Alcohol Coating Increased Magnetic Nanoparticle Dispersion in Poly(glycerol sebacate)

2.1 Introduction

Magnetic nanoparticles (MNPs) have been demonstrated wide application capabilities in various fields, including magnetic fluids [59], cell labeling [60], magnetic resonance imaging [61], hyperthermia treatment [25] and catalyst for environmental remediation [62]. In most of the above-described applications, MNPs was reported to perform the best when the size is below a critical value, which is material-dependent but is usually lower than 20 nm [63]. The MNPs with diameter less than 20 nm usually shows superparamagnetism at room temperature, which exhibits high constant saturated magnetization when exter-

nal magnetic field is applied while negligible remanence and coercivity when the external magnetic field is removed [63, 64].

However, an inevitable problem associated with MNPs is their intrinsic instability, which makes them easy to form agglomerates to reduce the surface energy associated with the high surface area to volume ratio. In addition, bare MNPs are highly active and will be gradually oxidized when exposed in air, which may result in loss of magnetism and dispersibility [63]. Thus, protection strategies were developed to reduce aggregation and chemically stabilize the bare MNPs by creating an electrostatic double layer or by shifting the isoelectric point [65]. These protection layers or coatings are composed of many different materials, including organic substances such as poly(lactic-co-glycolic acid) (PLGA) [66], poly(ethylene glycol) (PEG) [67], polyvinyl alcohol (PVA) [68], et al., and inorganic substances such as silica [65, 69] et al. Among those coating materials, polymers are usually used for biomedical applications. However, most of the polymers require organic solvent for use, which is difficult to accomplish in MNPs synthesis process.

Polyvinyl alcohol is an inexpensive polymer that is water-soluble, biodegradable and biocompatible [70]. Reports have shown that PVA can be ideal coating materials for MNPs in biomedical applications [71]. In this case, a further study on the weight percent of PVA that should be used for the coating is necessary to determine the least amount of PVA that can help achieve the best dispersion and least agglomeration in polymer solutions.

In this study, we synthesized MNPs with different weight percent of PVA coatings, and the PVA-coated MNPs were characterized with various methods. The PVA-coated

MNPs were also dispersed in polymer solution to determine the ideal weight percent of PVA coating for homogeneous distribution of MNPs in polymer substrates, which will benefit the future synthesis of magnetic nanocomposites with desired polymer.

2.2 Materials and Methods

2.2.1 Synthesis of PVA-Coated Magnetic Nanoparticles

Pure magnetic nanoparticles (MNPs) with different weight percent of polyvinyl alcohol (PVA) as the surface modifier were synthesized using the co-precipitation method modified from our previous work [72]. Briefly, deionized water (DI water; Milli-Q Integral Water Purification System, EMD Millipore, Billerica, MA) was deoxygenated by bubbling with nitrogen for 40 minutes. Then different amount of PVA (#341584, molecular weight 89000-98000, Sigma-Aldrich, St. Louis, MO) was dissolved in same volume of deoxygenated DI water in separate containers under the temperature of 100 °C for 30 minutes to make 0, 1.37, 2.33, 4.92, 11.06 and 18.96 mg/mL PVA solution. The weight percent of PVA to the final PVA-coated MNPs were set to 0, 3, 5, 10, 20 and 30%. Based on the calculation of the chemical reaction, proper amount of FeCl_3 (#169430010, Sigma-Aldrich, St. Louis, MO) and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (#44939, Sigma-Aldrich, St. Louis, MO) powder with a molar ratio of 2:1 was then dissolved in each PVA solution and stirred at room temperature for additional 30 minutes. A $25\frac{1}{2}$ gauge needle was used to gradually drop the aqueous mixture of PVA/ FeCl_3 / $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ into 50 mL of 10%-diluted ammonium hydroxide solution (#338818, Sigma-Aldrich, St. Louis, MO) to produce magnetic nanoparticles. MNPs were

washed with deoxygenated DI water for five times, dried in a vacuum oven under 80 °C for 3 hours, and ground using a mortar and pestle.

2.2.2 Characterizations of PVA-Coated MNPs

The surface morphology of MNPs was examined using a scanning electron microscope (SEM; Nova NanoSEM450, FEI). MNPs were mounted onto the SEM sample holder using copper tape. An acceleration voltage of 10 kV and Everhart-Thornley secondary electron detector was used for SEM imaging. The sizes of each group of MNPs were analyzed based on the high-resolution SEM image using the quantitative analysis tools of ImageJ software, with which 100 MNPs were measured and the average Feret diameter was calculated with an assumption that the synthesized MNPs were spherical. The crystal structure of the MNPs was analyzed using X-ray diffraction (XRD; PANalytical X-ray diffractometer, Empyrean) at 45 kV and 40 mA with a 0.026° step size. The magnetization of the MNPs was tested with a magnetometer (7400 Series VSM, Lake Shore Cryotronics, Inc. Henderson, NV) in the testing field range of -10,000 to 10,000 gauss and a hysteresis loop was obtained for analysis.

2.2.3 Dispersion of PVA-coated MNPs in Polymer Solution

Poly(glycerol sebacate) (PGS) (RegenerezTM RG-100, Secant Medical, Inc., Telford, PA) was dissolved in 100% ethanol to make PGS solution with concentration of 361.1 mg/mL. Each group of MNPs with amount of 1 mg was dispersed separately in same volume of PGS solution to make homogeneous suspensions. The suspensions were then

separated into two groups, with one group used to coat the whole surface of microscopic glass slides with size of 7.5×2.5 cm for optical imaging using a microscope (Nikon Ti-S eclipse, Nikon, Japan), and another group used to coat the whole surface of round-shaped cover glass with diameter of 12 mm (#22-293232, Fisher Scientific, Hampton, NH) for photographic imaging using a digital camera (Model: EOS Rebel T3i Digital SLR, Canon USA, Inc., Melville, NY) and absorbance test using a micro-plate reader (Model Infinite M200 Pro, Tecan, Switzerland). Bare glass slides and pure PGS solution on glass slide were used as reference groups.

2.3 Results

2.3.1 SEM Characterization of PVA-Coated MNPs

The synthesized PVA-coated MNPs all exhibited spherical morphology, as shown in the scanning electron micrograph (SEM) at a magnification of $50,000\times$ (Figure 2.1). Quantitative analysis of the SEM image of nanoparticles showed that the average diameter of the MNPs was 5-10 nm. And the diameter of each MNPs group was labeled on Figure 2.1(A-F). TEM image of 30% PVA-coated MNPs showed lattice planes and the lattice spacing was measured to be 0.487 nm, which is indexed to the (111) plane of Fe_3O_4 crystal structure [73].

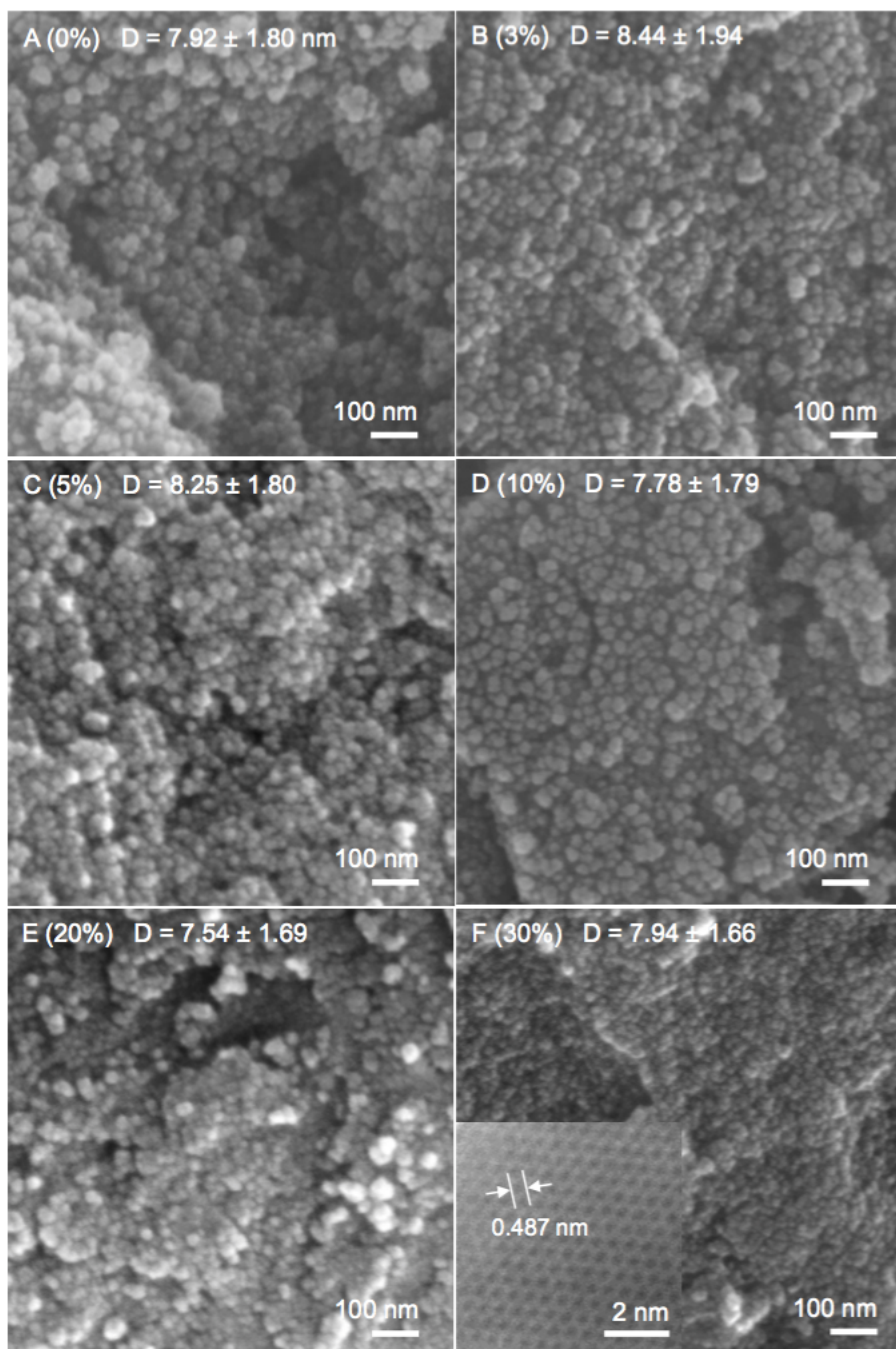


Figure 2.1: SEM images of magnetic nanoparticles coated with increasing weight percent of PVA. Each group of magnetic nanoparticle was referred to by the weight percent of PVA used in the synthesis. SEM images show the sphere morphology and size of magnetic nanoparticles coated with (A) 0%, (B) 3%, (C) 5%, (D) 10%, (E) 20% and (F) 30% of PVA. Original magnification = 50,000 $\times$, scale bar = 100 nm. Inset in (F): high-magnification TEM image showing the lattice planes. The interplanar distance was measured to be 0.487 nm, which is indexed to be the (111) plane of Fe_3O_4 crystal structure. Original magnification = 740,000 $\times$, scale bar = 2 nm.

2.3.2 X-Ray Diffraction Pattern of PVA-Coated MNPs

XRD pattern confirmed that the synthesized nanoparticles were iron oxide and the major peak positions for all the groups were identical (Figure 2.2). Although Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ are very close to each other per the standard XRD pattern (PDF #19629 and #39-1346, respectively), the major peaks for Fe_3O_4 were present while the minor peaks for $\gamma\text{-Fe}_2\text{O}_3$ were absent, indicating the synthesized nanoparticles were most likely to be Fe_3O_4 phase.

2.3.3 Magnetization of PVA-Coated MNPs

The hysteresis loop of different groups of MNPs showed similar pattern, with coercivity less than 20 gauss and saturated magnetization ranging from 40 to 60 emu/g without obvious relationship with PVA weight percent (Figure 2.3). The low coercivity after the removal of external magnetic field is in the range usually considered to be superparamagnetic [6]. Superparamagnetism is the property that the magnetization of MNPs can be saturated under an external magnetic field, but in the absence of this field their net magnetic moments are often randomized to zero by thermal agitation [64].

2.3.4 Dispersion of PVA-Coated MNPs in PGS Solution

Optical images showing the same amount of 0%, 3%, 5%, 10%, 20% and 30% PVA-coated MNPs dispersed in PGS. Both the white and black dots presented in the images are MNPs or the aggregates of MNPs due to the difference of the focus plane (Figure 2.4).

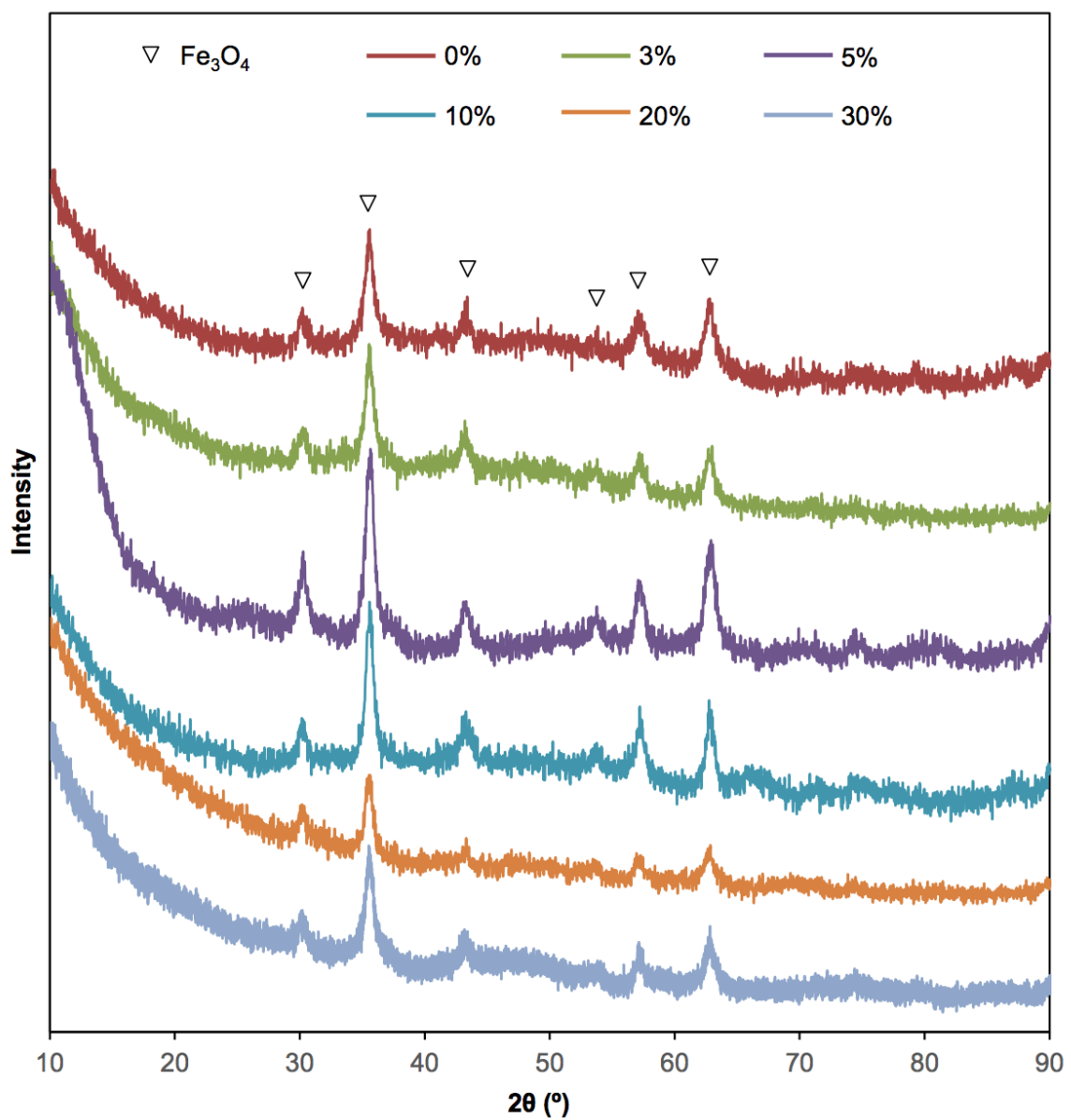


Figure 2.2: X-ray diffraction patterns of the magnetic nanoparticles coated with increasing weight percent of PVA. Step size = 0.026° . Fe_3O_4 standard pattern JCPDS #65-3107.

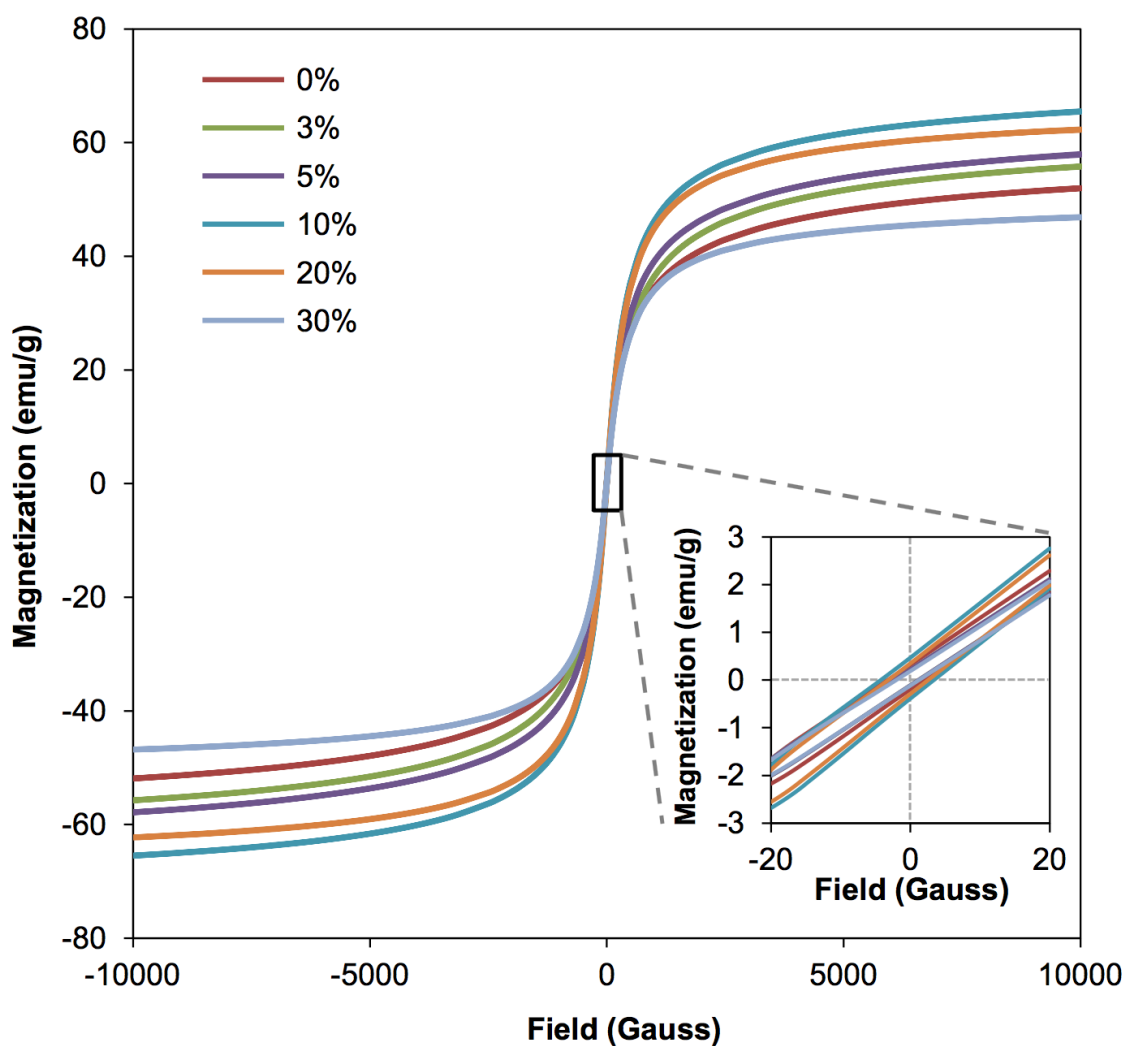


Figure 2.3: Hysteresis loop of the magnetic nanoparticles coated with increasing weight percent of PVA. Inset: magnetization of different groups of magnetic nanoparticles at the applied field ranging from -20 Gauss to 20 Gauss, showing the property of low coercivity and superparamagnetism.

Images showed that with the increasing weight percent of PVA coating, the visibility of PVA-coated MNPs in PGS decreased.

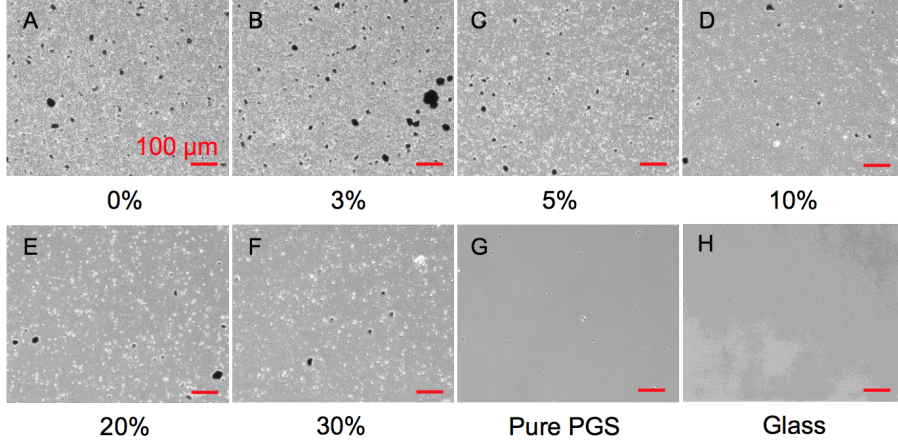


Figure 2.4: Optical images showing the same amount of (A) 0%, (B) 3%, (C) 5%, (D) 10%, (E) 20% and (F) 30% PVA-coated magnetic nanoparticles dispersed in PGS, and optical images of (G) pure PGS without particles and (H) glass reference. Scale bar = 100 μm .

Photographic images showed that with the increasing weight percent of PVA coating, PVA-coated MNPs dispersed in PGS showed a color transition from dark brown to clear white similar to pure PGS solution (Figure 2.5). Absorbance test results were presented with transmittance value. The transmittance spectra of the same amount of 0, 3, 5, 10, 20 and 30% PVA-coated MNPs dispersed in PGS showed that with the increasing weight percent of PVA coating, the transmittance of each group increased from 26.8% to 82.7% at the wavelength of 340 nm and from 60.8% to 98.4% at 900 nm, which indicated an increase of corresponding transparency (Figure 2.5). The transmittance of PVA-coated MNPs showed a clear separation from the transmittance of pure MNPs. The relationship of the transmittance among all the groups is $0\% < 3\% < 5\% < 10\%, 20\% < 30\% < \text{glass}$

< pure PGS. The trend of the transmittance is consistent at all wavelengths tested and confirmed the optical imaging and photographic imaging results.

2.4 Discussion

SEM images showed that small and sphere shaped magnetic nanoparticles were produced with different weight percent of PVA coating, and each group of MNPs exhibited similar size, crystallinity, low coercivity and superparamagnetism. This indicates that the weight percent of PVA coating does not have significant influence on the size, crystallinity and magnetism of the MNPs synthesized with co-precipitation method. However, the weight percent of PVA coating does have influence on the dispersion of MNPs in polymer solution. Optical images, photographic images and transmittance spectra showed that the dispersion quality increased with an increasing weight percent of PVA coating. In this study, 30% PVA-coated MNPs group showed the lightest color and possessed the highest transmittance in the dispersion of different groups of MNPs in PGS solution.

As Seget et al. has mentioned in their study of colloidal stability, they explained that agglomeration can be identified "by eye" and when a suspension turns turbid, it indicates the occurrence of a weak flocculation/aggregation of the nanoparticles, and vice versa [74]. In this study of the PVA-coated MNPs, the suspension of MNPs in PGS was less turbid with a higher weight percent of PVA coating, which indicated less formation of aggregates.

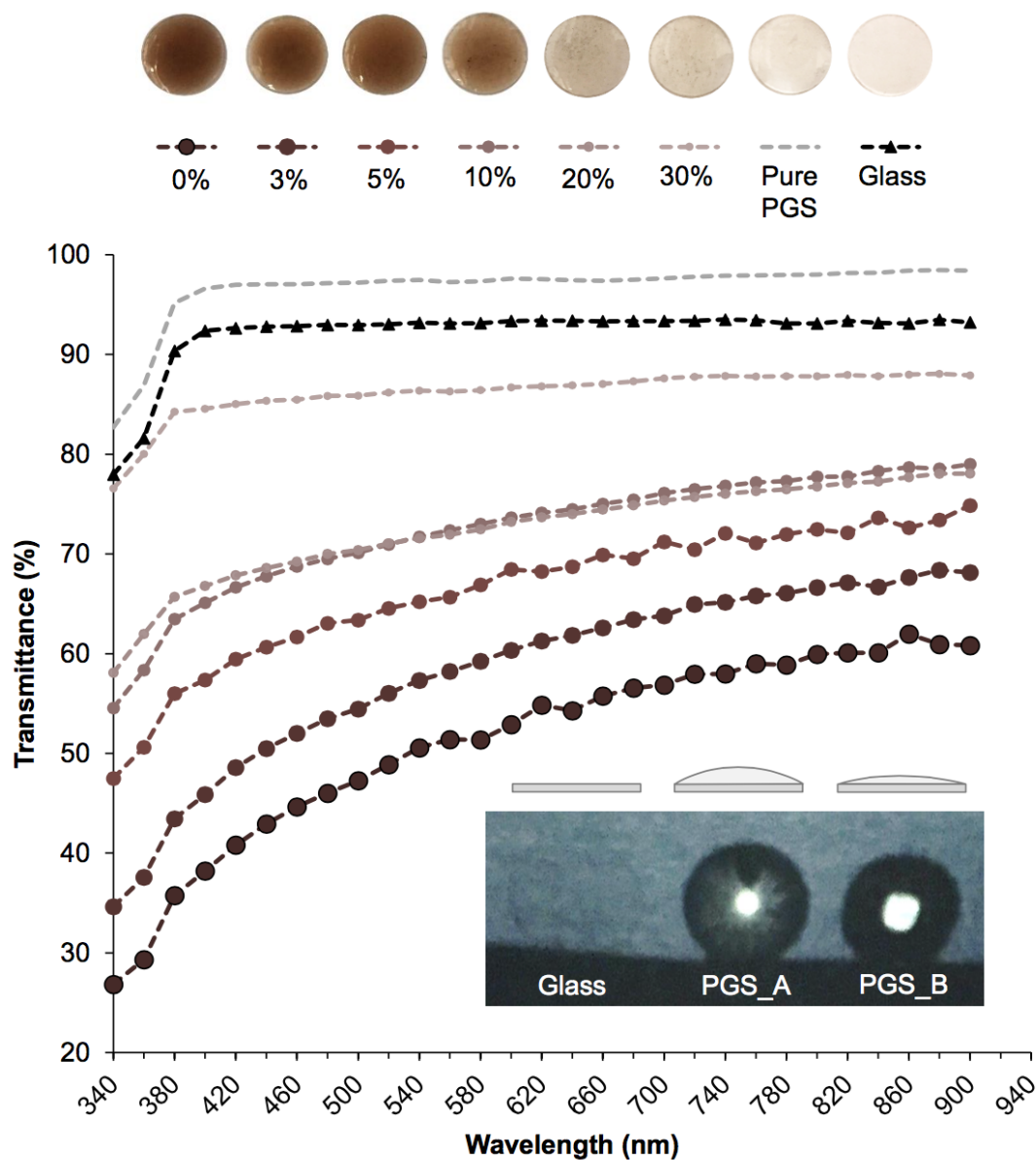


Figure 2.5: Transmittance spectra of the same amount of 0, 3, 5, 10, 20 and 30% PVA-coated magnetic nanoparticles dispersed in PGS, pure PGS without particles and glass reference. Photos in legend: showing the color transition from dark brown of 0% PVA-coated MNPs dispersed in PGS, to lighter brown of 30% PVA-coated MNPs dispersed in PGS, to clear white of pure PGS without particles and glass reference. Inset: comparison of light passes through glass, PGS_A (36 mg PGS deposited on glass) and PGS_B (18 mg PGS deposited on glass), showing the ability of PGS concentrating light.

Theoretically, with the increasing weight percent of PVA coating, the volume of the PVA coating should increase and the volume of the Fe_3O_4 core should decrease per unit mass of each group of MNPs. The calculated overall volume of PVA-coated MNPs increased from $1.934 \times 10^4 \text{ cm}^3/\text{mg}$ of pure MNPs to $3.875 \times 10^4 \text{ cm}^3/\text{mg}$ of 30% PVA-coated MNPs, thus, when the total mass of the PVA-coated MNPs are the same, the 30% PVA-coated MNPs have the largest volume. When particles are dispersed in polymer solution and if all groups have the same dispersibility, the 30% PVA-coated MNPs should prevent more light from passing through the sample and result in the lowest transmittance. However, the test result that 30% PVA-coated MNPs group has the highest transmittance indicates that it has the best dispersibility among all MNPs groups. Furthermore, even if the 30% PVA-coated MNPs has the best dispersibility among all the groups, the MNPs are still optically detectable, which makes it less transparent than the polymer solution alone.

In addition to the 30% PVA-coated MNPs, the fact that pure PGS coated cover glass is more "optically transparent" than the cover glass alone is also of interesting. Thus, two groups of different amount of PGS coated cover glass and the cover glass alone were tested with a optical light source and the corresponding image after light passing through was recorded with a camera (Model: EOS Rebel T3i Digital SLR, Canon USA, Inc., Melville, NY), and the image is shown in the inset of Figure 2.5. It can be seen that the cover glass alone is almost optically transparent while the cover glass coated with PGS showed light concentration at the center of the samples. The cover glass coated with more PGS showed light gathering close to a half-convex lens, and the cover glass coated with less PGS showed

light gathered all at the central area. This result is consistent with the surface morphology of the sample, that when the surface of cover glass is coated with a thin layer of PGS, coated layer would form an arc with a large curvature, and when the surface of cover glass is coated with a thicker layer of PGS, the coated layer would form an arc with a smaller curvature. Thus, refraction of light passing through the cover glass and PGS coating layer would be different and exhibit various optical phenomena. Due the mechanism of the micro-plate reader, the concentrated light at the center would be detected and result in an increase of the transmitted light intensity and subsequent transmittance. However, this does not apply to the cover glass coated with PGS dispersed with PVA-coated or non-coated MNPs because those MNPs would scatter the light significantly and diminish the half-convex lens effect.

2.5 Conclusions

This study has reported the synthesis and characterization of 0, 3, 5, 10, 20 and 30% PVA-coated magnetic nanoparticles. Study showed that all groups of MNPs exhibited sphere shape with diameter of 5-10 nm, similar crystallinity and superparamagnetism. The size, shape, crystallinity and magnetism of MNPs are not affected by the weight percent of PVA coating, indicating the preservation of particle properties. However, with higher weight percent of PVA coating, less aggregates of MNPs were formed and better dispersion in polymer solution were achieved. Thus, in future studies, 30% PVA-coated magnetic

nanoparticles should be used for the synthesis of magnetic nanocomposites for homogeneous dispersion in polymer substrates.

2.6 Acknowledgements

We would like to thank Mohammed Aldosary and Dr. Jin Shi for their kind help with hysteresis loop test. We thank Central Facility for Advanced Microscopy (CFAMM) at the University of California, Riverside, for the use of SEM, and Ilkeun Lee and Dr. Krassimir Bozhilov for the training on SEM. We thank Dr. Mustafa Ozgul for the help with TEM.

Chapter 3

Magnetic nanocomposite hydrogel for Potential Cartilage Tissue Engineering: Synthesis, Characterization, and Cytocompatibility with Bone Marrow Derived Mesenchymal Stem Cells

3.1 Introduction

Currently, natural and synthetic biocomposites, e.g., collagen-PLGA composites [75], hyaluronic acid-chitosan composites [76], have been studied for regeneration of cartilage tissue. In addition to the chemistry of the biomaterials, the structure of the scaffold at the nano- and microscale is also vital for cartilage regeneration. Nanomaterials such as

nanofibers [77] and materials with nanostructured surfaces [78, 79] have been explored to mimic the extracellular matrix (ECM) of cartilage. However, nanostructures developed so far still need further improvement to closely mimic the chemistry and the nano-to-micro structures of native cartilage, induce desirable cellular responses for cartilage regeneration, and provide tunable mechanical properties similar to the ECM.

In recent decades, magnetic nanoparticles have attracted growing interests for biomedical applications. For example, magnetic nanoparticles have been explored extensively as contrast agents to enhance magnetic resonance imaging (MRI) for medical diagnosis [80], as drug carriers to improve controlled drug delivery to targeted sites [81], or as hyperthermia agents for cancer treatment [82]. Nevertheless, magnetic nanoparticles alone may not be able to provide optimal properties. Integration of magnetic nanoparticles with other desirable components is an attractive approach to create nanocomposites with integrated properties for biomedical applications. For example, Meenach et al. studied UV-polymerized iron oxide nanoparticles and poly(N-isopropylacrylamide) (PNIPAAm) nanocomposites for drug delivery and hyperthermia treatment for cancers; their results showed favorable NIH3T3 murine fibroblasts viability, indicating minimal cytotoxicity [83]. In a few recent studies, magnetic nanoparticles have been incorporated into scaffolds for bone tissue engineering. For instance, Bock et al. transformed standard commercial scaffolds made of hydroxyapatite and collagen into magnetic scaffolds by dip-coating them in aqueous ferrofluids. The results showed that the magnetic nanoparticles were integrated into the porous structures of the scaffolds, and the resulted magnetic scaffolds supported

adhesion and proliferation of human bone marrow derived mesenchymal stem cells (BMSCs) in vitro [84]. Zhang et al. developed a three-dimensional magnetic composite scaffold made out of magnetite (Fe_3O_4), mesoporous bioactive glass (MBG), and polycaprolactone (PCL) using a 3D Bioplotter. They reported that the nanocomposite scaffold significantly stimulated human BMSCs proliferation, alkaline phosphatase (ALP) activity, osteogenic gene expression, and ECM mineralization [85].

However, magnetic nanocomposites for soft tissue engineering, e.g., articular cartilage, remains largely unexplored, even though using magnetic nanoparticles or a magnetic field for cartilage tissue engineering showed promising results. Specifically, Kobayashi et al. magnetically labeled human BMSCs using magnetic nanoparticles (ferumoxides) and showed promising results for delivering the cells to degenerated human cartilage to form ECM in vitro [86]. Moreover, electromagnetic fields (EMF) have been shown to increase the expression of type II collagen, thus improving chondrogenic differentiation of human BMSCs and stimulating chondrogenesis [87].

Building on these promising results, we took a new direction of designing magnetic nanocomposite hydrogel scaffolds to harvest the beneficial properties of magnetic nanoparticles while providing a biomimetic, bioactive, and biodegradable platform for potential cartilage tissue engineering. Specifically, we synthesized a novel magnetic nanocomposite hydrogel (MagGel) by mechanical dispersion of magnetic nanoparticles into a hybrid hydrogel (referred to as Gel). The Gel is composed of type II collagen (Col II), hyaluronic acid

(HA), and polyethylene glycol (PEG); all these components are biodegradable and provide beneficial properties for cartilage regeneration.

Natural components of Col II and HA are intended to mimic ECM of natural cartilage. Specifically, Col II is the primary protein in hyaline cartilage ECM, where a dense network of fine collagen fibrils provide tensile strength to the tissue [88]. Hyaluronic acid (HA) is a nonsulfated glycosaminoglycan [89] and a major structural component of the ECM in cartilage, which provides compressive strength for cartilage and maintains the ordered structure of collagen [88, 90]. HA is known to interact with chondrocyte cell surface receptors such as CD44, which influences endocytosis of HA, and this in turn influences cellular functions [91]. It was reported that the benzyl ester of HA enabled expression of cartilage-specific Col II from chondrocytes, and immobilization of HA onto the surface of poly(D,L-lactic acid-co-glycolic acid) scaffolds enhanced adhesion and proliferation of bovine chondrocytes [92, 93]. Thus, HA is one of the key factors for cartilage repair due to its ability to support cartilage mechanical strength and influence cellular functions.

The natural polymers of Col II and HA components promote cellular adhesion due to their bioactivity and permit enzymatic degradation, while the multiarmed poly(ethylene glycol) (PEG) component provides the tunable control over physical, chemical, and mechanical properties. PEG is hydrolytically degradable with well documented biocompatibility for cartilage tissue engineering [94, 95]. PEG-based hydrogels can be seeded with cells homogeneously and then photopolymerized to form scaffolds in situ without damaging living cells [96]. In contrast to natural polymers, PEG alone lacks the biological recognition due

to its bioinert nature and thus does not provide an ideal environment for cell adhesion and tissue formation [97]. Copolymerization of PEG with other polymers or monomers can address the inherent limitations of PEG and provide tunable properties. For example, Pfister et al. reported a PEG-dimethacrylate (PEGDMA)-based hydrogel with adjustable shear moduli from 10 kPa to 1 MPa that can mimic mechanical properties of cartilage by altering the PEGDMA concentration [98]. Hutson et al. copolymerized PEG with methacrylated gelatin, and the resulted hydrogel improved fibroblast surface binding when compared with PEG alone [99]. To mimic hyaline cartilage closely, we rationally designed 4-arm-PEG to be copolymerized with Col II and HA, the main proteins in natural cartilage.

The objective of this study was to synthesize a biodegradable and bioactive MagGel scaffold composed of magnetic nanoparticles, Col II, HA, and PEG through chemical cross-linking and mechanical dispersion for potential cartilage tissue engineering applications. We characterized the microstructure and composition of the MagGels, evaluated their magnetic responses and degradation properties in physiological fluids, and investigated their cytocompatibility with BMSCs within 24 h. To our knowledge, this is the first report of designing a bioactive and biodegradable magnetic nanoparticle/organic composite for cartilage tissue engineering. The MagGel developed in this study has a promising potential for cartilage tissue engineering applications.

3.2 Materials and Methods

3.2.1 Synthesis and Characterization of MagGel

Synthesis and Characterization of Magnetic Nanoparticles

Magnetic nanoparticles with poly(vinyl alcohol) (PVA) as the surface modifier were synthesized using the coprecipitation method modified from the procedure reported in the literature [70]. Briefly, PVA (#341584, molecular weight 89 000-98 000, Sigma-Aldrich, St. Louis, MO) was dissolved in deionized water (DI water; Milli-Q Integral Water Purification System, EMD Millipore, Billerica, MA) under the temperature of 100 °C for 30 min to make 50 mg/mL PVA solution. FeCl_3 (#169430010, Sigma-Aldrich, St. Louis, MO) and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (#44939, Sigma-Aldrich, St. Louis, MO) powder with a molar ratio of 2:1 was then dissolved in the 50 mg/mL PVA solution and stirred at room temperature for an additional 30 min. A 25-gauge needle was used to gradually drop the aqueous mixture of PVA/ FeCl_3 / $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ into 15 mL of 10%-diluted ammonium hydroxide solution (#338818, Sigma-Aldrich, St. Louis, MO) to produce magnetic nanoparticles. The magnetic nanoparticles were washed with DI water for three times, dried overnight, and ground using a mortar and pestle.

The surface morphology and elemental composition of the magnetic nanoparticles (MNPs) were examined using a scanning electron microscope (SEM; Nova NanoSEM450, FEI) and the attached detector for energy dispersive X-ray spectroscopy (EDS; X- Max 50 silicon drift detector), respectively. The magnetic nano- particles were mounted onto the

SEM sample holder using the copper tape. An acceleration voltage of 10 kV and Everhart-Thornley secondary electron detector was used for SEM imaging, and an acceleration voltage of 20 kV and EDS detector was used for elemental composition analysis. The size of nanoparticles was analyzed on the basis of the high-resolution SEM image using the quantitative analysis tools of ImageJ software. The Feret diameter of 200 magnetic nanoparticles was measured using ImageJ, and the average Feret diameter was calculated with an assumption that the synthesized MNPs are spherical. Alternatively, the size and distribution of magnetic nanoparticles were analyzed using a laser diffraction-based technique (Beckman Coulter LS 13 320 Particle Size Analyzer, Beckman Coulter, Inc., Brea, CA). Briefly, a suspension with 10 mg/mL of magnetic nanoparticles was prepared by dispersing magnetic nanoparticles into DI water; the suspension was then added into the universal liquid module of the Beckman Coulter particle size analyzer, further sonicated to improve dispersion, and analyzed using laser diffraction. The crystal structure of the magnetic nanoparticles was analyzed using X-ray diffraction (XRD; PANalytical X-ray diffractometer, Empyrean) at 45 kV and 40 mA with a 0.02° step size.

Extraction and Characterization of Cartilage and Collagen

Subchondral bone plugs with hyaline cartilage (0.5 cm in diameter) were harvested from the stifle joints of two-year-old sheep using an osteochondral autograft transfer system (Arthrex OATS, Arthrex, Inc., Naples, FL). The bone plug was photographed using a camera (Coolpix L22 12.0 MP, Nikon, Japan). The bone plug was then prepared for SEM

imaging through rapid freezing in liquid nitrogen. The surface and cross-section of the bone plug was examined using an environmental SEM (Hitachi TM-1000 Tabletop Scanning Electron Microscope, Hitachi High-Technologies America, Inc.).

Type II collagen (Col II) was extracted from the harvested cartilage following a protocol modified from the procedure reported in the literature [100]. Briefly, stifle joints of two-year-old sheep were harvested and dissected to expose articular cartilage. The articular cartilage surface was rinsed with 70% isopropyl alcohol (#I9030, Sigma-Aldrich, St. Louis, MO) followed by phosphate buffered saline (PBS; #2810305, MP Biomedicals, LLC, Solon, OH). A scalpel was used to scrape the articular cartilage from the femoral condyle, tibia plateau, and patella. The cartilage pieces were minced in ice-cold 0.025 M Tris(hydroxymethyl) aminomethane (Tris buffer, #252859, Thermo Fisher Scientific, Inc., Waltham, MA) to improve Col II extraction. The cartilage was mixed on a rotator (#11-402-12, Fisher Scientific, Waltham, MA) overnight in a solution of 2.5 M guanidine (#BP178- 500, Thermo Fisher Scientific, Inc., Waltham, MA) in 0.025 M Tris buffer at 4 °C. The cartilage was washed three times with cold DI water for 30 min at 4600g and 4 °C to remove guanidine. The supernatant was removed, and the cartilage was resuspended in 500 mM acetic acid (#320099, Sigma-Aldrich, St. Louis, MO) and adjusted to pH 2.8. Next, 1 g of pepsin (#P7000, Sigma-Aldrich, St. Louis, MO) was added for every 20 g of cartilage, and the suspension was mixed for 48 h at 4 °C. The suspension was centrifuged for 30 min at 5000g at 4 °C, and the supernatant was collected. Then, 5 M NaCl (#S3014, Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 0.8 M, and collagen

was allowed to precipitate for 24 h. The precipitated collagen was collected by centrifuging at 4600g for 60 min at 4 °C and resuspended in 100 mM acetic acid overnight at 4 °C. The collagen solution was dialyzed against PBS overnight at 4 °C. The extracted collagen was lyophilized and stored at 20 °C until use.

Extracted type II collagen was characterized using Fourier Transform Infrared Spectroscopy (FTIR, Nicolet 6700 FTIR spectrometer, Thermo Fisher Scientific Inc., Waltham, MA). Briefly, potassium hydroxide (KOH) was heated overnight to remove residual water. The mixture of collagen and KOH was ground using a mortar and pestle and pressed into a pellet at 10,000 psi. The translucent pellet was placed in the FTIR sample holder and scanned at the wavenumbers between 4000 and 500 cm^{-1} .

Synthesis of the MagGel and Gel

The hybrid gel (Gel) was synthesized through chemical cross-linking, modified from the process reported by Calderon et al [101]. Figure 3.1 shows the chemical structures of the representative Col II fragment and other major components used in the Gel synthesis. Briefly, 1-ethyl-3-(3-(dimethylamino)propyl)- carbodiimide·HCl (EDC·HCl, hereafter referred as EDC, #22980, Thermo Fisher Scientific Inc., Waltham, MA) was dissolved in 2-(N- morpholino)ethanesulfonic acid (MES buffer, #28390, Thermo Fisher Scientific Inc., Waltham, MA) by vortex to obtain a concentration of 19 mg/mL as the cross-linking reagent. HA (#HA200 K-1, Lifecore Biomedical, LLC, Chaska, MN) and Col II (extracted from sheep femur condyle and tibia plateau) were added into EDC solution followed by the

addition of extra MES buffer to help dissolve HA and Col II. Col II and HA had a weight ratio of 9:1 to mimic cartilage ECM. Afterward, 4-arm-PEG (#4arm-NH2-10K, JenKem Technology, Allen, TX) was added to achieve the Col II/HA/PEG weight ratio of 9:1:2 and mixed by vortex and sonication. The mixture was then divided into two separate microcentrifuge tubes; magnetic nano- particles were added into one tube to produce the MagGel (A), while the other tube was left alone for the formation of the Gel (B). Additional MES buffer was added into each tube and sonicated to help dissolve 4-arm-PEG. In the (A) MagGel, the dry weight ratio of magnetic nanoparticles to the Col II/HA/PEG gel was 1:24. N-Hydroxysuccinimide (NHS, #24500, Thermo Fisher Scientific Inc., Waltham, MA) was dissolved in MES buffer and added to A and B, respectively, to induce further cross-linking and produce stable Col II/ HA/PEG hydrogel with or without magnetic nanoparticles. A and B solution were then transferred into the wells of a 48-well plate and incubated at 37 °C for 24 h. Afterward, additional EDC was added to each solution and incubated in a 37 °C incubator overnight to further cross-link Col II, HA, and 4-arm-PEG. The resulted MagGel and Gel samples had a similar dimension and volume to be consistent for in vitro degradation and cell study.

Characterizations of the MagGel in Comparison with the Gel

The MagGel and Gel were dehydrated in a series of ethanol solutions with concentrations of 30%, 50%, 70%, 80% 90%, 95%, and finally 100%, 1 h in each concentration of ethanol. The dehydration in 100% ethanol was repeated three times. The samples were

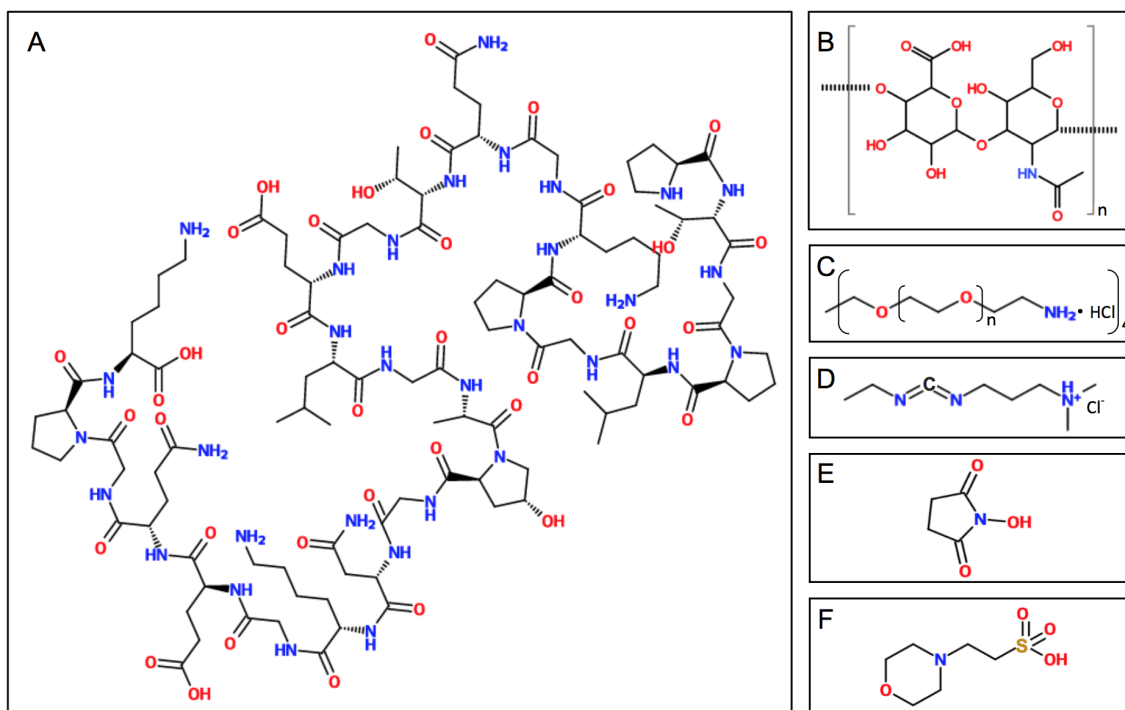


Figure 3.1: Chemical structures of each component used for synthesizing the magnetic nanocomposite hydrogels (MagGel) and the hybrid hydrogels (Gel). (A) Representative type II collagen fragment (CAS No. 144703-90-2), (B) HA, (C) 4-arm-PEG, (D) EDC·HCl, (E) NHS and (F) MES buffer.

then transferred to a 50 mL conical tube with the tube screwed lightly onto the cap and kept inverted. The inverted tube was dipped into liquid nitrogen for 30 s. After that, the samples were kept in the cap of the conical tube and covered with Parafilm M (#PM992, Bemis Flexible Packaging, Neenah, WI) with a few punctures generated by a 25 G needle. The covered cap was loaded into a freeze-dry-system (cat# 7934026, FreeZone Plus 6 Cascade Console Freeze-Dry System, Labconco, Kansas City, MO) and left overnight. The freeze-dried sample was characterized using FTIR (Nicolet 6700 FTIR spectrometer, Thermo Fisher Scientific Inc.). The microstructure of the lyophilized samples was observed using SEM, and the elemental composition was quantified using energy dispersive X-ray spectroscopy (EDS; AZtec, Oxford instruments).

3.2.2 Magnetic Response of the MagGel and Magnetic Nanoparticles

The response of the MagGel and magnetic nanoparticles to the external magnetic field was tested using a N42 neodymium magnet (#DEX0, K&J Magnetics, Inc., Pipersville, PA) with a diameter of 22 mm and a height of 25 mm. MagGel with a diameter of 11 mm and a height of 3 mm was immersed in a Petri dish filled with MES buffer, and the neodymium magnet was placed 3 cm away from the right side of the hydrogel. The magnet was slowly moved toward the hydrogel, and magnetic response was recorded. To study remote magnetic guidance, MagGel was immersed in a Petri dish filled with MES buffer, and the neodymium magnet was applied above the dish to direct the MagGel to move from one side of the dish to the other side. The diameter of the dish was 8 cm, and the movement

of the MagGel guided by the external magnet was recorded. The vertical and horizontal movement of MagGel in response to the magnet was tested in a 15 mL centrifuge tube filled with MES buffer. In the vertical movement test, after the MagGel settled to the bottom of the centrifuge tube by gravity, the magnet was placed outside the tube and moved up against gravity. For the horizontal test, the centrifuge tube was placed horizontally and the magnet was applied on the side of the tube to direct the MagGel to move horizontally from one end to the other end of the tube. The whole process of vertical and horizontal movement was recorded. In addition, the response of magnetic nanoparticles to the magnet was recorded separately. A small amount of magnetic nanoparticles weighing 1 mg was mechanically dispersed in 1 mL of DI water using vortex. The magnet was placed about 5 mm away from the microcentrifuge tube, and the time needed to collect the dispersed magnetic nanoparticles toward the magnet was recorded. Please see Supplemental Videos 1 and 2 for these experiments on magnetic responses.

3.2.3 Degradation Study of the MagGel versus Gel

The MagGel was stored in MES buffer before the degradation study in the revised simulated body fluid (rSBF). The rSBF had the same ionic concentration as human blood plasma, containing Na^+ (142.0 mM), K^+ (5.0 mM), Mg^{2+} (1.5 mM), Ca^{2+} (2.5 mM), Cl^- (103 mM), HCO_3^- (27 mM), HPO_4^{2-} (1.0 mM), and SO_4^{2-} (0.5 mM) ions. MES buffer was removed at the beginning of the degradation study, and 5 mL of rSBF was added to the Petri dish to submerge the MagGel sample. The samples were incubated in an incubator

at 37 °C, and 1 mL of rSBF was added to the Petri dish every day to compensate the fluid loss due to evaporation. The lyophilized Gel was incubated in a phosphate buffered saline (PBS) following a similar procedure. PBS was composed of 137 mM sodium chloride, 2.68 mM potassium chloride, 1.47 mM KH_2PO_4 , and 8.1 mM Na_2HPO_4 , with corresponding ions of Na^+ (153.2 mM), K^+ (4.2 mM), Cl^- (139.7 mM), HPO_4^{2-} (8.1 mM), and $\text{H}_2\text{PO}_4^{4-}$ (1.5 mM). The testing samples were weighed every day until they lost structural integrity.

3.2.4 *In Vitro* Cytocompatibility Study with BMSCs

Preparation of BMSC culture

Dulbeccos modified Eagles medium powder (DMEM, #SLBC9050, high glucose, D5648, Sigma-Aldrich, St. Louis, MO) and sodium bicarbonate (NaHCO_3 ; #S5761, Sigma-Aldrich, St. Louis, MO) were dissolved in DI water to obtain a media with DMEM and NaHCO_3 concentration of 13.4 and 3.7 mg/mL, respectively. The pH of media was adjusted to 7.1 by adding 1 M hydrogen chloride (HCl) solution. The media were sterilized using a 0.2 μm filter (#596-0020, Thermo Fisher Scientific, Inc., Waltham, MA). For BMSC culture, the media were supplemented with 10% fetal bovine serum (FBS; HyClone, #SH30910, Thermo Fisher Scientific Inc., Waltham, MA) and 1% penicillin/streptomycin (P/S; HyClone, #SV30010, Thermo Fisher Scientific, Inc., Waltham, MA). Hereafter, DMEM + NaHCO_3 + 10% FBS + 1% P/S is referred to as DMEM. BMSCs were harvested from the marrow cavity of the femur and tibia of 3-week-old Sprague-Dawley rat weanlings immediately after euthanasia by CO_2 asphyxiation. The protocol for harvesting BMSCs was

approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California at Riverside and established previously in our laboratory [102]. Briefly, the distal and proximal ends of the harvested femur and tibia were dissected, and the bone marrow was flushed out of the marrow cavity and collected using DMEM. The collected bone marrow was filtered through a 70 μm nylon strainer (#22363548, Thermo Fisher Scientific, Inc., Waltham, MA) to remove cell aggregates and tissue debris. The filtered cells were cultured in DMEM under standard cell culture conditions (i.e., 37 °C, 5% CO₂/ 95% air, humidified, sterile environment) to 90-95% confluency prior to the in vitro study with the materials of interest.

Sample Preparation for Cell Culture

MagGel and Gel samples were cut into quarters for BMSC study. Three pieces of MagGel and three pieces of Gel were sterilized by soaking in 70% ethanol solution for 20 min. Magnetic nanoparticles were placed in a microcentrifuge tube and exposed to ultraviolet radiation for 2 h to disinfect. Glass slides with a dimension of 1 cm $\times$ 1 cm were cut from Fisher premium microscope slides (#12-544-1, Thermo Fisher Scientific, Inc., Waltham, MA). Six glass slides were cleaned with acetone and 70% ethanol solution using a bath sonicator for 1 h each and then exposed to ultraviolet radiation for 2 h to disinfect. Three glass slides were used as substrates to support magnetic nanoparticles; magnetic nanoparticles on glass slides were used as the particle control. The other three glass slides were used as a reference group for BMSC culture.

After sterilization, three pieces of MagGel, three pieces of Gel, and six pieces of glass slides were placed into separate wells of a 12-well tissue culture-treated plate. MagGel and Gel samples were placed directly into the culture well without the glass slides. Magnetic nanoparticles of 2.5 mg were suspended in 40 μ L of DMEM homogeneously. Each 10 μ L of the magnetic nanoparticle suspension was gently pipetted onto each of the three glass slides. The mass of magnetic nanoparticles in each well was the same as that in each piece of MagGel. Each well was gently rinsed with 2 mL of PBS to equilibrate the well and samples before seeding cells. The neodymium magnet was used to prevent aspiration of magnetic nanoparticles during rinsing steps.

BMSC Culture with the Samples

BMSCs were washed with PBS and detached from the culture flasks using trypsin (#25200-056, Life Technologies, Carlsbad, CA). BMSCs in each flask were collected into a 15 mL-centrifuge tube and centrifuged for 5 min at 1000 rpm. After the supernatant was removed, the BMSCs were resuspended in fresh media. Cell suspension was mixed with trypan blue solution (#T8154, Sigma-Aldrich, St. Louis, MO) at a dilution factor of 2, and the cell number was counted using a hemocytometer (#1475, Reichert Bright-Line, Hausser Scientific, Horsham, PA). The volume of cell suspension needed for seeding at a density of 10,000 cells/cm² was calculated on the basis of the cell count. BMSCs were diluted to the needed concentration using fresh DMEM, and then, 2 mL of the diluted cell suspension was pipetted into each well of the 12-well plate for the culture of cells on the surface of the gels.

The culture plate was gently placed back into the incubator and cultured for 24 h under the standard cell culture conditions.

Characterization of BMSC Adhesion and Morphology

After 24 h, the media were collected from each well and the samples were moved to a new 12-well plate. Nonadherent cells were washed away using PBS. Adhered cells on the 12-well plate or on the samples were fixed with 4% paraformaldehyde (PFA; #15714-S, Electron Microscopy Science, Hatfield, PA) solution, stained with Alexa Fluor 488 Phalloidin (#A12379, Life Technologies, Carlsbad, CA) for F-actin and 4',6-diamidino-2-phenylindole (DAPI; #D3571, Life Technologies, Carlsbad, CA) for cell nuclei. After fixing and staining, cells were imaged using a fluorescence microscope (Nikon Ti-S eclipse, Nikon, Japan), and the images were merged using ImageJ. Cell adhesion density (cells/cm²) was calculated for each sample. Briefly, five random spots in each well were imaged, and the number of cells in each image was quantified. The cell density in each image area (or spot) was calculated using eq 1, and the average cell density in each well was calculated using eq 2.

$$\text{cell density in spot } i = \frac{\text{cell number in image } i}{\text{area of image } i} \quad (1)$$

$$\text{average cell density} = \frac{\sum_1^i \text{cell density in spot } i}{5} \quad (2)$$

3.2.5 Statistical Analysis

Experiments were run in triplicate, and the data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukeys Honest Significant Difference (HSD) posthoc test. Statistically significant difference was considered at $p < 0.05$.

3.3 Results

3.3.1 Characterization of Materials of Interest

Characterization of Magnetic Nanoparticles

The synthesized magnetic nanoparticles exhibited spherical morphology, as shown in the scanning electron micrograph at a high magnification of $100\,000\times$ (Figure 3.2A). The corresponding EDS analyses (Figure 3.2B) showed the presence of iron (Fe; 24 at. %), oxygen (O; 49 at. %), carbon (C; 26 at. %) and a trace amount of chlorine (Cl; 1 at. %). As expected, C was detected due to the PVA surface modifier. The trace Cl might be a result of residual reactant that was not completely washed away after the synthesis; more thorough washes will be helpful in the future research. Quantitative analysis of the SEM image of nanoparticles showed that the average diameter of the magnetic nanoparticles was 15 ± 5 nm (Figure 3.2C), while the particle analysis result from laser diffraction indicated the average diameter of magnetic particles to be 77 ± 4 nm (Figure 3.2D). This difference observed was most likely due to nanoparticle agglomeration during laser diffraction measurement. XRD pattern confirmed that the synthesized nanoparticles were iron oxide (Figure 3.2E). The major XRD peaks for Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ are very close to one another according to

the standard XRD pattern (PDF #19629 and #39-1346, respectively). As shown in Figure 3.2E, major peaks for Fe_3O_4 were present, and the minor peaks for $\gamma\text{-Fe}_2\text{O}_3$ were absent, indicating the synthesized nanoparticles were most likely to be in the Fe_3O_4 phase.

Characterization of Articular Cartilage and Extracted Collagen

The surface and cross-section of the dissected bone plug were characterized using SEM (Figure 3.3). Figure 3.3A showed the sheep femur condyle where the bone plugs were harvested. Figure 3.3B showed the side of the bone plug used for SEM characterization. Figure 3.3C showed the overview of the zonal transition in the bone plug from the articular surface to mineralized subchondral bone. Figure 3.3C₁ - C₆ provided a magnified view for each transition area between the articular cartilage to subchondral bone. Figure 3.3C₁ showed the smooth surface of articular cartilage. Figure 3.3C₂ - C₄ showed the microstructural changes in collagen network as we gradually moved the area of observation from articular cartilage surface to subchondral bone. Figure 3.3C₅, C₆ showed increasing particulate mineral content toward the cartilagebone interface, correlated to the changes in mechanical properties between cartilage and bone.

Type II collagen extracted from the sheep femur condyle or tibia plateau was characterized using FTIR, as shown in Figure 3.4. The FTIR spectrum for type II collagen from femur condyle cartilage showed clear peaks for amide A, I, II, and III at 3420, 1653, 1560, and 1229 cm^{-1} , respectively (Figure 3.4A). Amide A band is due to the NH stretching

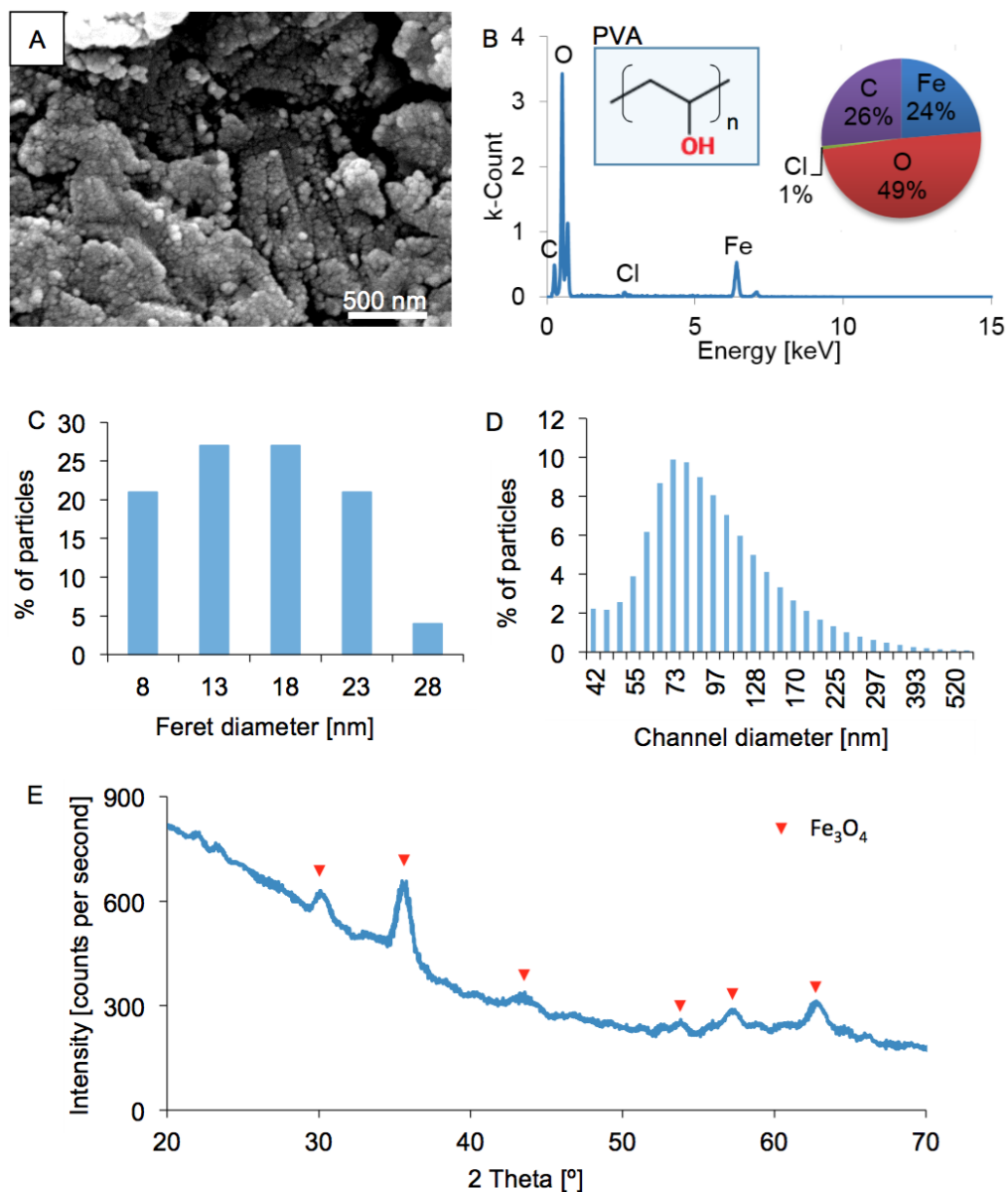


Figure 3.2: Characterization of magnetic nanoparticles. (A) SEM image. Scale bar = 500 nm. Original magnification = 100 000 $\times$. (B) EDS spectrum and quantification of elemental composition of magnetic nanoparticles in atomic percent (at. %). Inset: chemical structure of PVA. (C) Particle size and distribution based on quantitative analysis of SEM image in (A) using ImageJ software. (D) Particle size and distribution analyzed using laser diffraction. (E) XRD spectrum of magnetic nanoparticles.

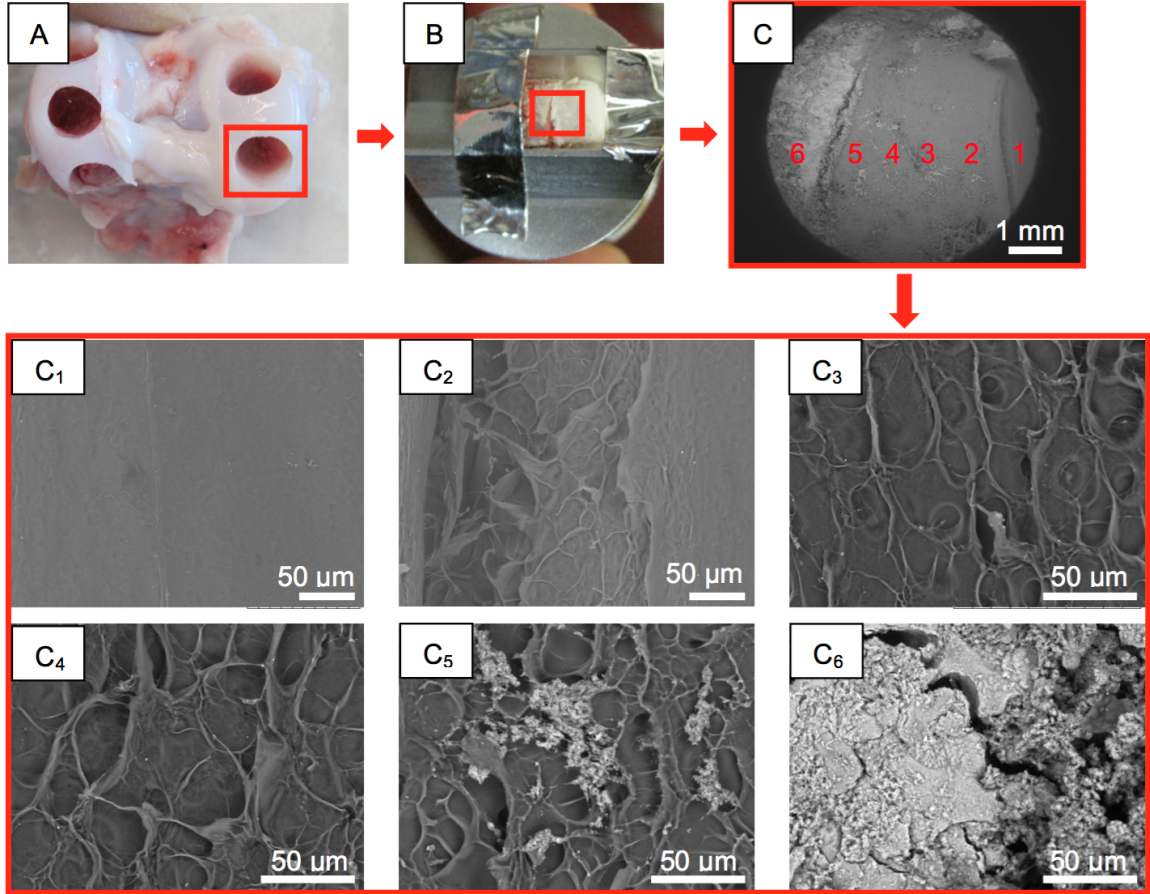


Figure 3.3: Characterization of cartilage tissue harvested from sheep femur condyle. (A) Photograph of sheep femur condyle where bone plugs were harvested. (B) Photograph of a harvested bone plug. (C) SEM image of the bone plug cross-section from articular surface to subchondral bone at low magnification. (C₁-C₆) Magnified SEM images of the transition area from articular surface to subchondral bone. Original magnification for C₁ and C₂ is 600 $\times$, and for C₃-C₆, it is 1000 $\times$. Scale bar = 50 μ m.

vibration in protein backbone, and amide I is the most intense absorption band in protein that is primarily dominated by the stretching vibrations of the C=O and C-N groups. The amide II and amide III bands are largely dependent on the N-H bending vibrations in protein. In comparison with the collagen from femur condyle cartilage, the collagen from tibia plateau cartilage showed a clear presence of amide A and I peaks at 3435 and 1661 cm^{-1} , respectively, but the peaks for amide II and III groups are smaller at 1559 and 1235 cm^{-1} , respectively (Figure 3.4A). Generally, the collagen from cartilage tissues at femur condyle or tibia plateau showed similar spectra.

The components for hydrogel synthesis, including HA, PEG, EDC, and NHS, were also characterized using FTIR. The broad peak centered around 3450 cm^{-1} is typical for O-H stretching vibration of HA and N-H stretching vibration of 4-arm-PEG. The relatively narrower peaks at 2897 cm^{-1} resulted from CH stretching vibration of both HA and 4-arm-PEG. The small peaks shown on HA spectrum at 1616 and 1566 cm^{-1} can be assigned to N-H bending, and the two peaks at 1410 and 1044 cm^{-1} are due to C-O-H stretching and C-O-C stretching vibrations, respectively [103]. The two adjacent peaks at 1650 and 1570 cm^{-1} are due to the N-H stretching vibration in the 4-arm-PEG. The narrow peak on 4-arm-PEG spectrum at 1111 cm^{-1} can be assigned to the C-N stretching vibration, and the two small peaks at 951 and 843 cm^{-1} can be assigned to C-O-C asymmetric stretching vibration [104]. The first broadest peak at 1705 cm^{-1} on the EDC spectrum shows the N-H bending vibration, and the following region of multiple peaks from 1500 to 700 cm^{-1}

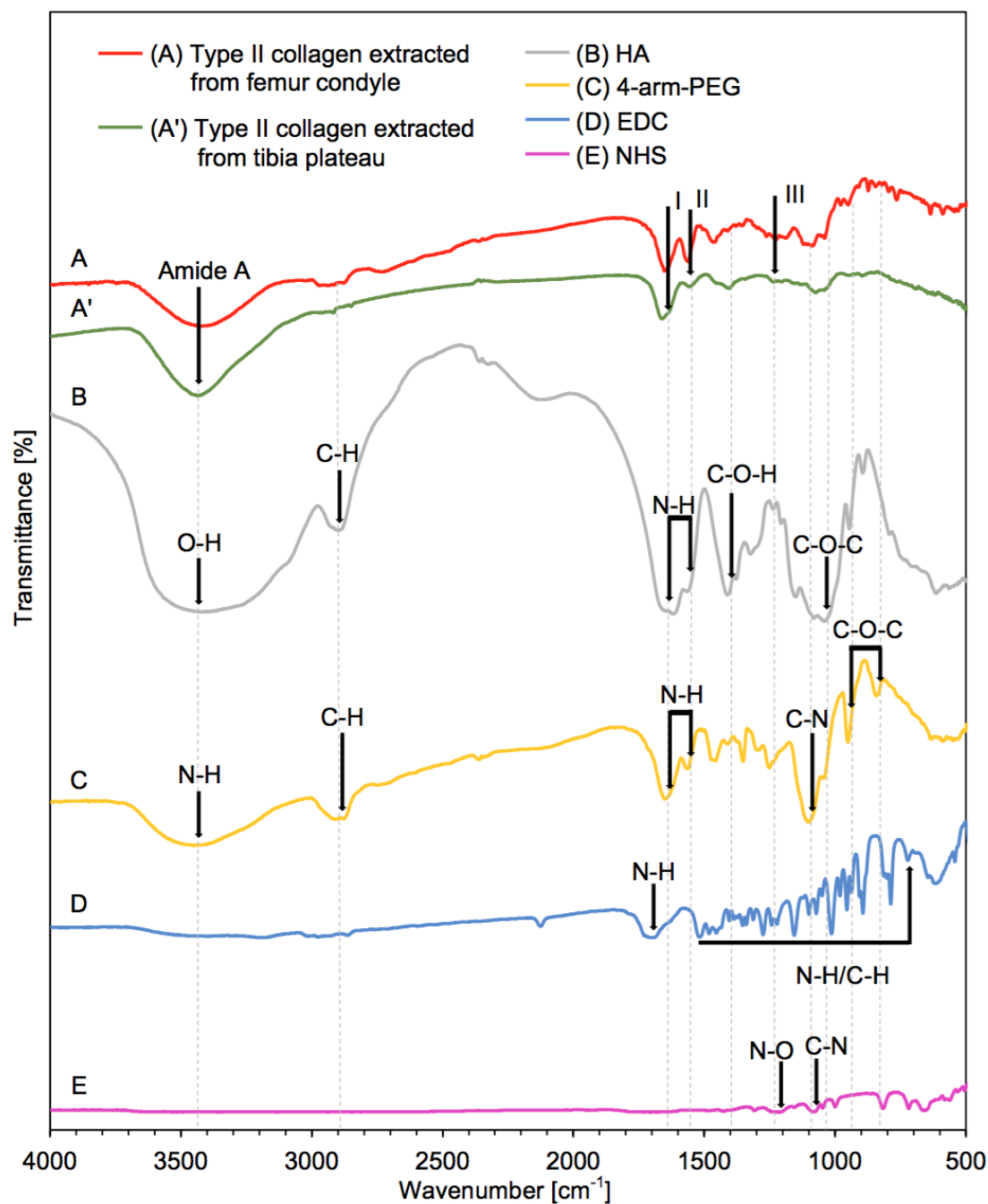


Figure 3.4: FTIR spectra of type II collagen extracted from sheep femur condyle or tibia plateau, and HA, 4-arm-PEG, EDC and NHS used for the synthesis of MagGel and Gel. (A) Type II collagen extracted from femur condyle, (A') type II collagen extracted from tibia plateau, (B) HA, (C) 4-arm-PEG, (D) EDC, (E) NHS.

can be assigned to C-N stretching or N-H bending vibrations [105, 106]. Compared with other components, the peaks on the FTIR spectrum of NHS are relatively smaller but still recognizable. The peak at 1213 cm^{-1} shows the N-O stretching vibration in NHS [107], and the peak at 1084 cm^{-1} represents the C-N stretching vibration.

Characterization of the MagGel in Comparison with the Gel

FTIR spectrum of MagGel was compared with that of the Gel in Figure 3.5. The FTIR spectra indicated that Type II collagen was incorporated since the peaks for amide A, I, II, and III were present, and the peak intensities of MagGel are greater than that of Gel. Significant differences in the FTIR spectra at wavenumber 945.8 and 842.7 cm^{-1} for the MagGel and Gel were observed; these peaks are due to C-O-C stretching and N-H bending vibrations, respectively. SEM images and EDS analyses of the lyophilized MagGel and Gel are shown in Figure 3.6. Both types of gels showed micron-scale porous structures, and the MagGel showed a rougher surface when compared with the Gel at a higher magnification of 10,000 (Figure 3.6A,B,D,E). The EDS analyses confirmed that the lyophilized MagGel contained C, N, O, and Fe (Figure 3.6C), while the Gel contained C, N, and O (Figure 3.6F). The gels were sputter coated with Pt/Pd prior to SEM and EDS analyses, which was excluded in quantification of elemental compositions.

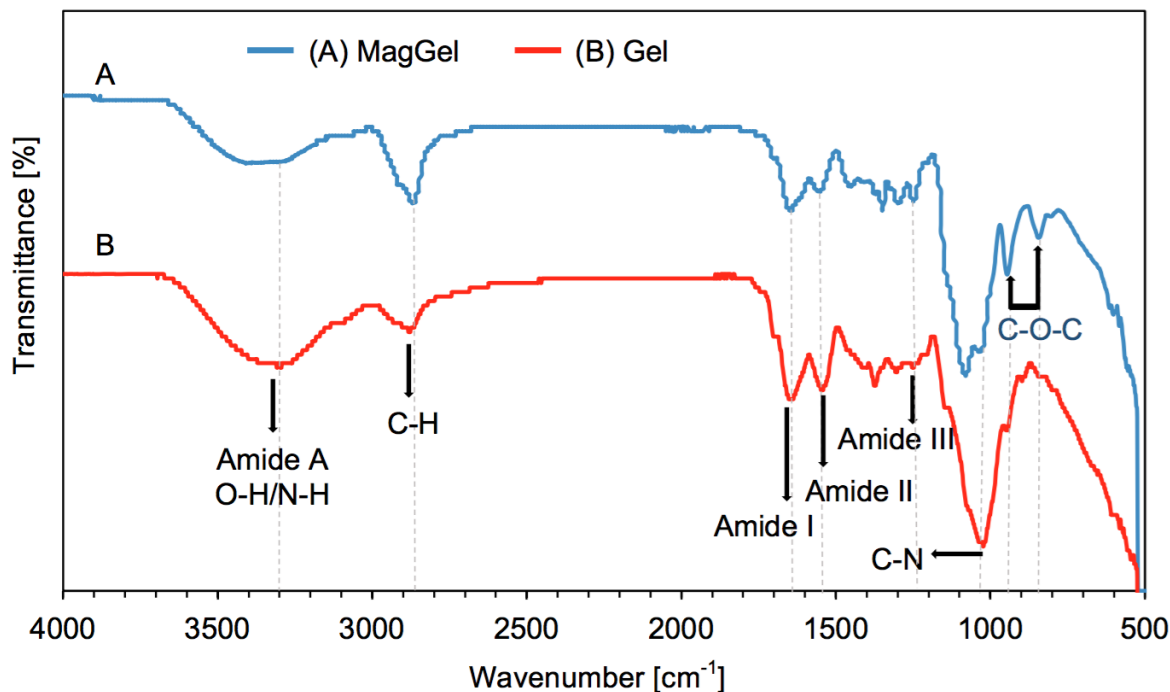


Figure 3.5: FTIR spectra of (A) MagGel and (B) Gel.

3.3.2 Magnetic Response in Aqueous Solutions

The MagGel showed magnetic response when exposed to an external magnet (Figure 3.7A). When the magnet was placed more than 22 mm away from the MagGel, there was no response. When the distance was reduced to 22 mm, the MagGel moved to the magnet in 1 s. In another test (see Supplemental Video 1), the diameter of the dish was 8 cm and the time for the MagGel to travel across this 8 cm distance was 3 s, so the approximate speed of the gel movement in MES buffer was 26.7 mm/s. The speed of the gel movement may be fine-tuned by adjusting the moving speed of the magnet and its magnetic intensity. The vertical movement test showed that the MagGel can be moved against gravity. The MagGel and magnet both had a cylindrical shape. Thus, the geometric center of the top

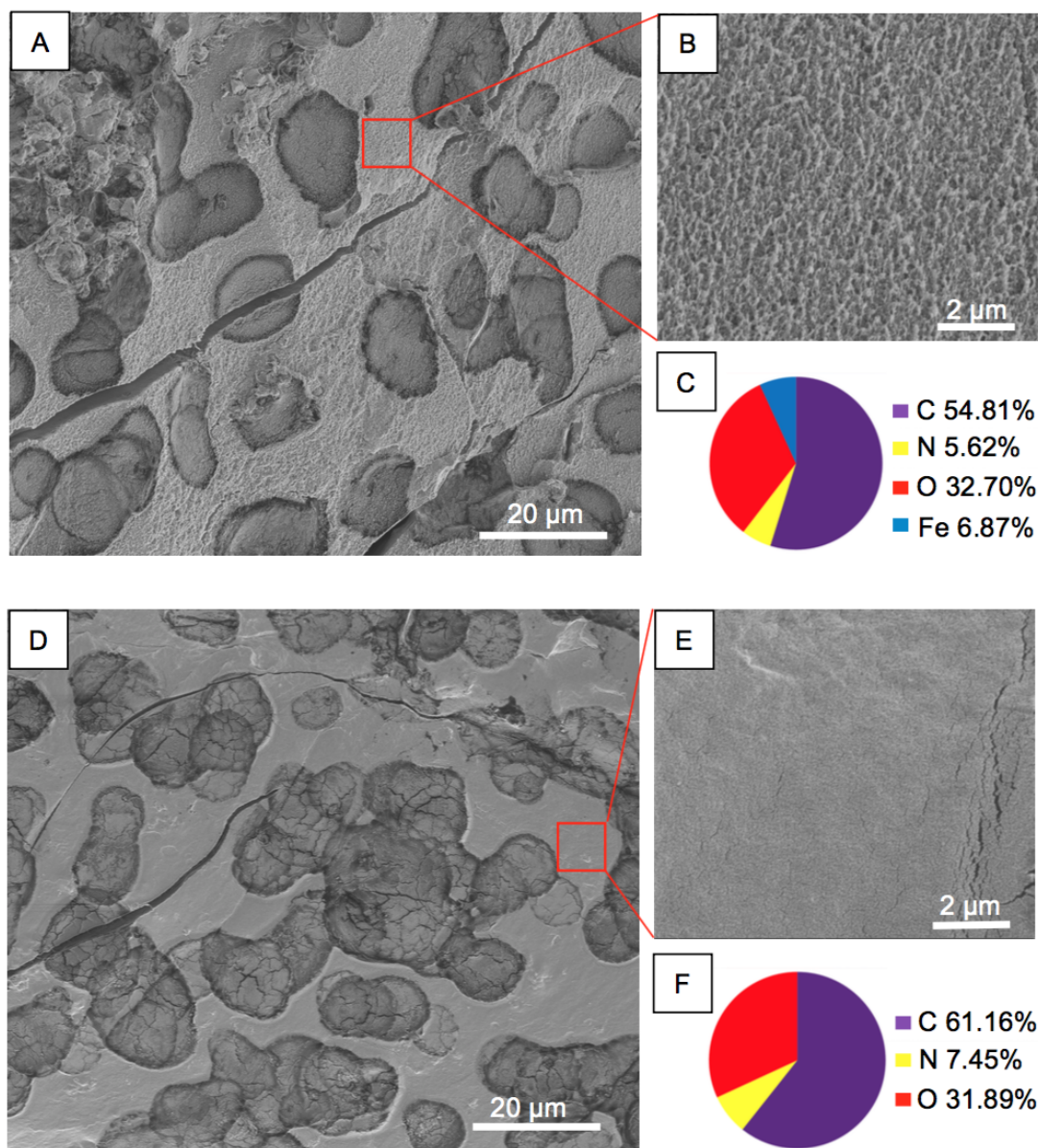


Figure 3.6: SEM images and EDS quantitative analysis (at.%) of (A, B and C) MagGel and (D, E and F) Gel. (A, D) low magnification SEM images; original magnification = 1000 $\times$. (B, E) high magnification SEM images; original magnification = 10,000 $\times$. (C, F) EDS elemental analysis of (A) MagGel and (D) Gel, performed at 1,000 $\times$.

or bottom cross section of MagGel had the strongest magnetic interactions with the top or bottom of the magnet, when compared with side-to-side magnetic interactions between the MagGel and the magnet. The horizontal movement of the same MagGel in the same centrifuge tube further confirmed this.

Figure refgels-movementB shows the response of magnetic nanoparticles to the same external magnet when suspended in DI water. When exposed to the magnet, the nanoparticles were attracted to the side of magnet placed 5 mm away within 3 s (Supplemental Video 2).

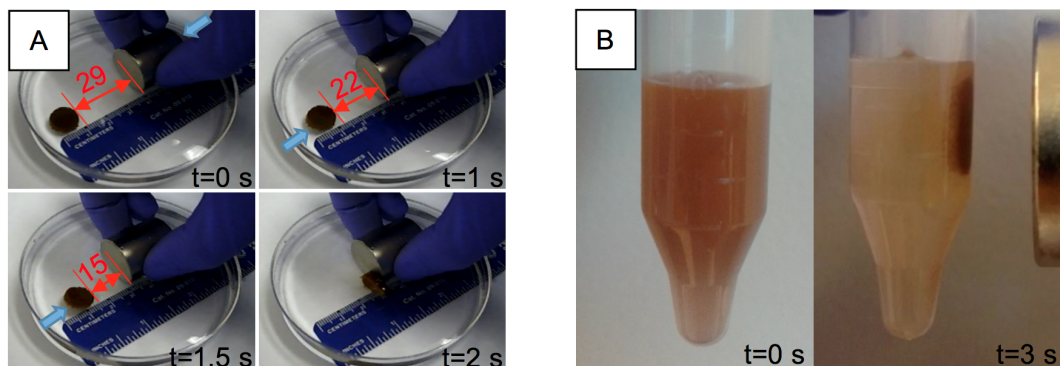


Figure 3.7: Magnetic responses of the synthesized MagGel and magnetic nanoparticles to a magnet. (A) MagGel in MES buffer (unit of numbers in red: mm) and (B) magnetic nanoparticles in DI water. Blue arrows indicate the object movement direction before next time point. Details are available in the supplemental video.

3.3.3 Degradation of the MagGel and Gel in Aqueous Solutions at 37 °C

Both MagGel and Gel absorbed water and swelled when they were cultured in SBF or PBS, as shown in Figure 3.8. Figure 3.8A shows the photographs of MagGel during the 21-day degradation study in SBF, and Figure 3.8B shows the degradation of

Gel in PBS. Both the MagGel and Gel lost structure integrity after 21 days of immersion in aqueous solutions at 37 °C. The surface morphology of both MagGel and Gel changed over time during degradation. The mass change over time confirmed water absorption during immersion in physiologically relevant fluids. Specifically, for MagGel, the initial wet mass was 400.2 mg and increased to 1402.2 mg on day 21, which was 3.5-fold of the initial mass. As for the Gel, after immersion, it became too soft to be picked up for the weight measurement without damaging the structure.

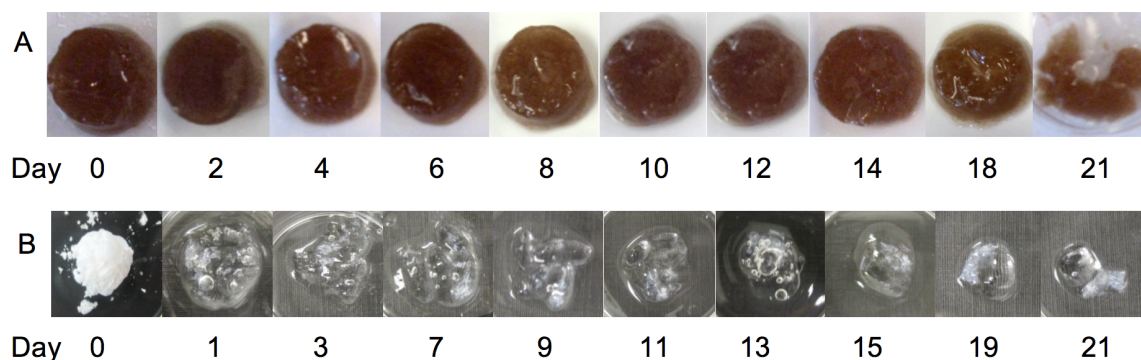


Figure 3.8: Macroscopic photographs of materials of interest during 21 days of degradation at 37 °C. (A) MagGel degradation in rSBF and (B) Gel degradation in PBS.

3.3.4 BMSC Adhesion and Morphology when Cultured with the MagGel, Gel, and Magnetic Nanoparticles

Figure 3.9 shows the adhesion and morphology of BMSCs after being cultured with MagGel, Gel, magnetic nanoparticles on glass slides, and a glass reference for 24 h. The BMSCs that adhered on the culture well with each sample were imaged. Surprisingly, both the MagGel and Gel samples lost structural integrity in BMSC culture and became

visually undetectable in the culture wells after 24 h. Thus, BMSCs could not be imaged on the samples. Interestingly, BMSCs engulfed nanoparticles and retained viability, as shown in Figure 3.9E, F. These magnetic nanoparticles appeared to be within the cytoplasm of the cells and close to the nuclei, but not in the cell nuclei. Some magnetic nanoparticles were also found to be outside of the cells. BMSC adhesion density in each well was quantified, as shown in Figure 3.10. Among all the groups, MagGel showed the highest average cell density, similar to the glass reference, but no statistically significant difference was detected. The Gel group showed the least cell density on average, less than the magnetic nanoparticle group, but no statistically significant difference was detected. The ANOVA analysis showed that there was no statistically significant difference among all the groups ($p = 0.0563 > 0.05$).

3.4 Discussions

3.4.1 Characterization of Magnetic Nanoparticles

The magnetic nanoparticle size was 15 ± 5 nm, as characterized by SEM (Figure 3.2A,C). The nanoparticles were attracted to the external magnet (Figure 3.7B), indicating their paramagnetic property. Paramagnetic property enables magnetic moments inside the magnetic nanoparticles to align in the same direction as the external magnetic field, and the nanoparticles would not retain any remnant magnetization upon removal of the external magnetic field. Paramagnetic property enabled magnetic nanoparticles to become dispersed

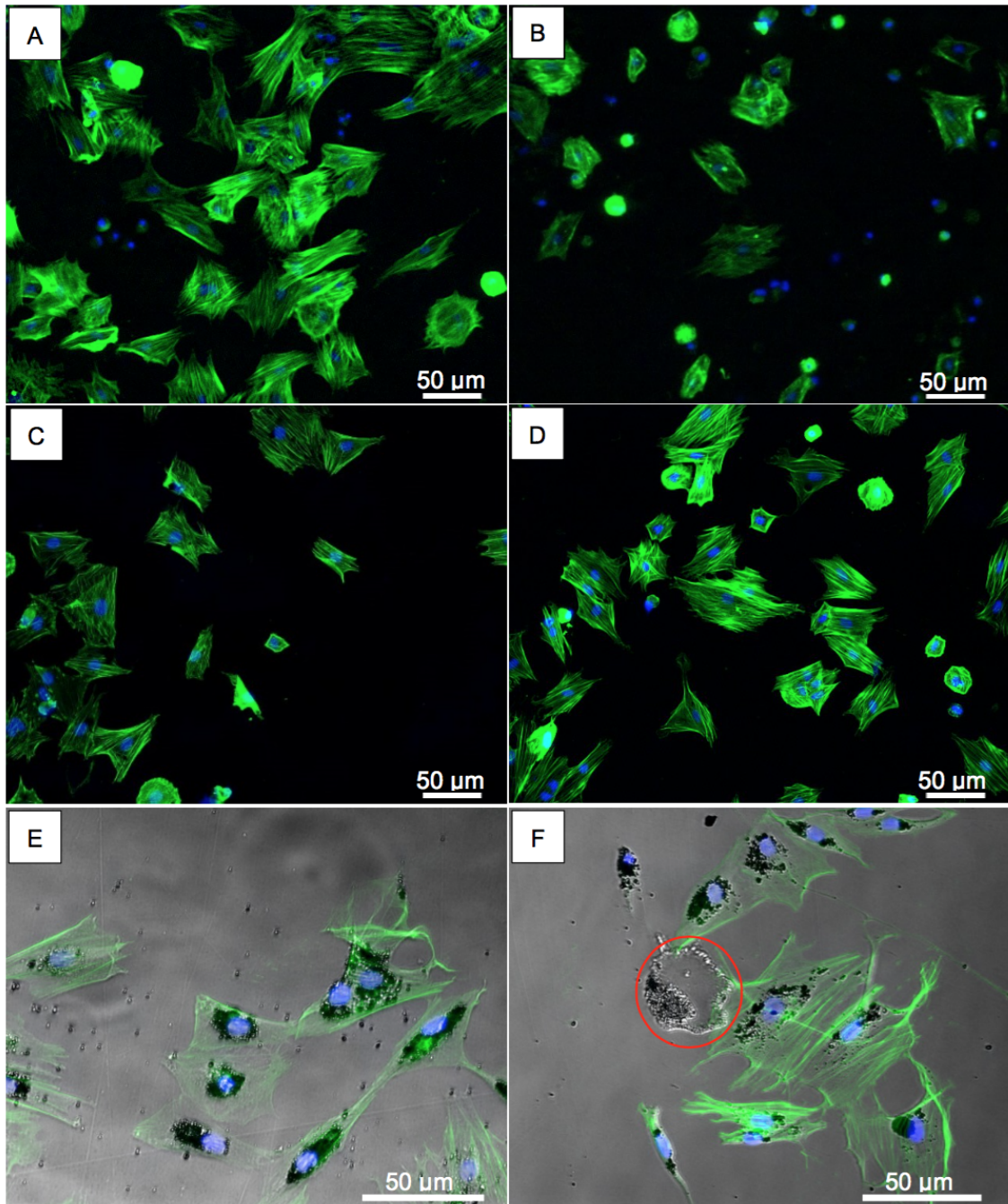


Figure 3.9: Fluorescence images of BMSC adhesion and morphology when cultured with materials of interest. (A) MagGel; (B) Gel; (C) magnetic nanoparticles on glass slides; (D) glass reference. (E, F) Fluorescence image overlay with bright field image, showing endocytosis of magnetic nanoparticles by BMSCs. Magnetic nanoparticles (black) intake by BMSCs did not enter nucleus (stained in blue). F-Actin of BMSCs were stained in green. Red circle highlights the magnetic nanoparticles outside of BMSCs. Scale bar = 50 μm .

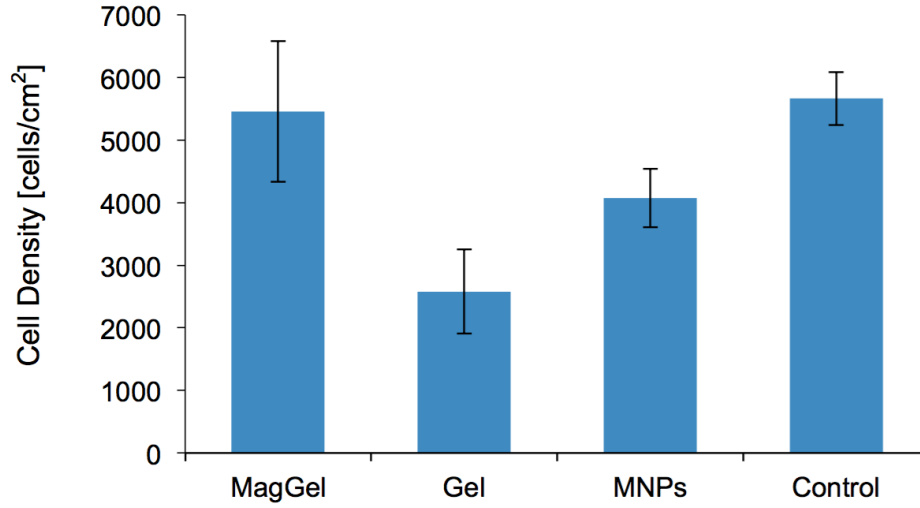


Figure 3.10: Cell density in each experimental group after 24 hours of culture. From left to right: BMSCs cultured with MagGel, Gel, magnetic nanoparticles (MNPs) and control group (i.e., glass reference). Values are mean $\pm$ standard error. $p = 0.0563$ for ANOVA.

again after removal of the magnetic field, which prevented them from agglomeration after exposure to the magnetic field. Paramagnetic property is beneficial in preventing nanoparticles from forming large agglomerates, and dispersed nanoparticles could be cleared out from the human body quickly after the degradation of MagGel [108]. However, particle size characterized using SEM and ImageJ (15 ± 5 nm) was much smaller than that characterized using laser diffraction (77 ± 4 nm), mainly due to the difference between the two measuring methods. When measuring particle size based on the SEM image, boundaries of individual magnetic nanoparticle were outlined and quantified using ImageJ, thus representing the actual size of each nanoparticle. In contrast, the magnetic nanoparticles formed agglomerates in DI water when they were injected into the laser diffraction particle analyzer, even though high power sonication was used to disperse these nanoparticles. On the basis of the principles of laser diffraction, individual magnetic nanoparticle versus particle agglomerates

could not be distinguished, and thus, both contributed to the overall particle size measurement. In other words, the larger particle size measured using laser diffraction was due to the presence of agglomerates.

EDS analysis (Figure 3.2B) showed the presence of Fe, Cl, O, and C in magnetic nanoparticles, which is expected from the source materials. During the synthesis, iron chloride reacted with ammonium hydroxide to form iron oxides ($\text{FeO} \cdot \text{Fe}_2\text{O}_3$). Specifically, Fe and Cl were from FeCl_3 and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, indicating a small amount of Cl (1 at. %) remained after the magnetic nanoparticles were washed with DI water for three times. PVA was also a possible source of the observed O peak, and it contributed to the C peak as well. EDS results showed the atomic ratio of Fe/O is 0.49, which was lower than the theoretical atomic ratio of 0.75 in Fe_3O_4 . However, since EDS analysis could not differentiate the O signals from PVA or iron oxide, it is necessary to confirm the phase of the nanoparticles using XRD.

The XRD pattern of synthesized magnetic nanoparticles (Figure 3.2E) showed the presence of major peaks for Fe_3O_4 , but absence of minor peaks for $\gamma\text{-Fe}_2\text{O}_3$, when compared with the standard XRD pattern of Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$, respectively. Combined with EDS analysis, we concluded that the nanoparticles were mostly in the form of magnetite Fe_3O_4 . It is still possible that these nanoparticles were partially oxidized to the form of $\gamma\text{-Fe}_2\text{O}_3$ considering their brown color appearing in DI water (Figure 3.7B). However, the presence of $\gamma\text{-Fe}_2\text{O}_3$ crystals did not have any substantial influence on the cytocompatibility of these magnetic nanoparticles with BMSCs within 24 h based on our in vitro results. Moreover, γ -

Fe_2O_3 was found to be cytocompatible with murine L929 fibroblasts [109], human umbilical vein endothelial cells [110], and HeLa cells [111].

3.4.2 Characterization of the MagGel and Gel

To synthesize the gels, we extracted Col II from sheep stifle joint. Prior to extraction, the stifle joint was characterized using SEM and we observed a clear transition from hyaline cartilage to mineralized subchondral bone (Figure 3.3). FTIR analysis of the extracted type II condyle and tibia collagen showed the presence of amide A, I, II, and III groups (Figure 3.4), which is consistent with the results reported by Barnes et al [100]. and other research groups [112, 113, 114]. FTIR spectra of the gels showed the conservation of amide A, I, II, and III bands of collagen as well as the C-H and C-N band of HA and 4-arm-PEG, respectively (Figure 3.5). The broad peak centered at 3300 cm^{-1} in both gels may also indicate the conservation of O-H stretching and N-H stretching vibrations in HA and 4-arm-PEG, respectively. The presence of C-O-C stretching at 945.8 and 842.7 cm^{-1} indicated the cross-linking of type II collagen with the 4-arm-PEG. The peaks of C-O-C are more apparent in the FTIR spectrum of MagGel than in the Gel. This might be caused by the presence of Fe. In another study, Abedini et al [68]. synthesized colloidal magnetite nanoparticles using γ radiation in an aqueous solution containing iron chloride in the presence of PVA. They also observed the presence of the small peak around 1100 cm^{-1} and attributed it to the presence of Fe ions.

In EDS analysis of the gels, the large amount of C and O and small amount of N were due to incorporation of Col II, HA, and PEG and the small amount of Fe in MagGel was from magnetic nanoparticles (Figure 3.6C, F). The incorporation of iron oxide did not affect the percentage of O but decreased the percentage of C and N in MagGel in a small range as compared with the Gel. The interaction of magnetic nanoparticles with other hydrogel components also increased the MagGel surface roughness compared to the Gel. Change of surface roughness through the addition of magnetic nanoparticles can also be seen in other studies [115]. In the SEM micrographs, circular-shaped dark areas were observed on the surface of both gels (Figure 3.6A, D). Both SEM visual observation and EDS analysis showed that the darker areas had similar features and composition as other areas (data not shown). This artifact might have been induced by the lyophilization process during which the water droplets were extracted from the gel matrix. Interestingly, the surface features on the lyophilized gels were similar to those surface features observed in the cartilage-bone transition area.

The degradation of MagGel and Gel was individually studied in rSBF and PBS and also monitored during in vitro culture with BMSCs in DMEM. After 24 h of culture in DMEM with BMSCs, both MagGel and Gel completely disintegrated and could not be collected from the culture. The faster gel degradation in DMEM with cells was likely due to the presence of excreted enzymes that could reduce the gel cross-linking and accelerate gel degradation.

3.4.3 Characterization of BMSC adhesion and morphology

The in vitro cell study showed that MagGel group had the greatest cell adhesion density on average when compared with the Gel group and magnetic nanoparticles group and a similar cell adhesion density as compared with the control, indicating good cytocompatibility within 24 h of culture. The ANOVA showed no statistically significant difference among all groups ($p = 0.0563 > 0.05$), but the p value was only slightly higher than the 0.05 significance level. It is possible that statistical significance might be detected with more repeats. Our hypothesis is that the synergistic effect of the hybrid gel and magnetic nanoparticles improved BMSC adhesion. First of all, the hybrid gel mimicked cartilage ECM, which provided a favorable environment for cells. Second, the interaction of magnetic nanoparticles with BMSCs might be beneficial for cell adhesion. Huang et al. demonstrated that the Fe_3O_4 nanoparticles increased human mesenchymal stem cells growth due to their ability to reduce intracellular H_2O_2 through intrinsic peroxidase-like activity. They also suggested that Fe_3O_4 nanoparticles could accelerate cell cycle progression [116]. Each magnetic nanoparticle could be considered as a small magnetic domain with a tiny magnetic field. When the magnetic nanoparticles are incorporated into the scaffold in a large number, the microenvironments inside or on the surface of the composite scaffold are composed of many tiny magnetic fields, and the total effect would likely be strengthened. As a result, the strengthened effect might affect ion channels on the cell membrane and initiate changes in cytoskeletal architecture under the magnetic field [117, 118]. For a time period shorter

than 24 h, we expect that the cell adhesion would be similar to the 24 h culture result since the cells usually adhere to a substrate 4 h after seeding and slowly adapt to the environment afterward. After 24 h, cells become more active in proliferation and migration. Besides, BMSCs have the potential to differentiate into chondrocytes, which would benefit the application of the hydrogels in cartilage tissue engineering. However, future research with a longer cell culture period is necessary to determine the long-term cytocompatibility of MagGel with BMSCs, proliferation and differentiation of BMSCs after 24 h, and the interaction mechanisms of MagGel with BMSCs.

Magnetic nanoparticle uptake by BMSCs was observed in cell culture. BMSCs maintained normal morphology with the magnetic nanoparticles in cytoplasm. Zhang et al [119]. reported that superparamagnetic magnetite nanoparticles (Fe_3O_4) could be taken up by mouse macrophage cells RAW 264.7 and breast cancer cells. Wilhelm et al [120]. showed that maghemite ($\gamma\text{-Fe}_2\text{O}_3$) exhibited a high level of cellular internalization by mouse macrophages, which was mediated by the affinity with cell membrane and endocytosis. Since Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ maghemite are very similar in structure and properties, we hypothesize that our synthesized magnetic nanoparticles were endocytosed as well. Besides, all the particles inside cells were distributed in cytoplasm but not in the nucleus, suggesting that either particle sizes were larger than the channel diameter of nuclear pore complexes (NPC) [121] or the control mechanism of NPC prevented the penetration of magnetic nanoparticles to protect cell genome [122]. The fact that the cell nuclei were particle-free might be the reason for the similar cell density when cultured with magnetic nanoparticles as compared

with the control, indicating good cytocompatibility within 24 h of culture despite the particle uptake by BMSCs. It is possible that the ingested magnetic nanoparticles would be broken down by lysosome and expelled from the cells through exocytosis [123]. However, further research is needed to determine the exocytosis process.

It has been reported that a low frequency electromagnetic field could enhance chondrogenesis in human adipose-derived stem cells [124] and in human mesenchymal stem cells [87]. Thus, it is hypothesized that integration of electromagnetic field with the MagGel could further control cell functions, e.g., directing adhesion, proliferation, and differentiation of BMSCs. The combined effects of external magnetic field and MagGel on cell functions should be further studied for potential cartilage tissue regeneration.

3.5 Conclusions

The MagGel synthesized in this study provides a promising scaffold for potential cartilage tissue engineering because the MagGel responded to an external magnetic field, which could be used to direct the scaffold to the exact cartilage defect site remotely using an external magnet. Moreover, the MagGel showed similar microstructure and chemistry as hyaline cartilage and was cytocompatible with BMSCs in vitro, as demonstrated by the increased BMSC viability when compared with the Gel in the 24 h culture. However, further cell studies with these gels in the presence of an external magnet are still needed to determine the combined effects of magnetic field and magnetic scaffold on cell functions. In addition, the presence of magnetic nanoparticles did not affect the viability of BMSCs

within 24 h of culture when compared with the control group. Although the hydrogels degraded much faster in DMEM with BMSCs than in rSBF or in PBS, further cross-linking of the gels could reduce the degradation rate of the hydrogels and improve the scaffold integrity for a longer period.

3.6 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b06939.

Supplemental video 1 showing the movement of MagGel in response to an external magnet (AVI)

Supplemental video 2 showing the movement of magnetic nanoparticles in response to an external magnet (AVI)

3.7 Acknowledgements

The authors thank Hellman Faculty Fellowship (H.L.) and the University of California Regents Faculty Fellowship (H.L.) for financial support. The authors thank U.S. National Science Foundation Graduate Research Fellowship Program for supporting the graduate student researcher (GRFP 2012125281 for J.L.) and California Institute for Regenerative Medicine (CIRM) Bridges to Stem Cell Research for supporting the undergraduate student researcher (A.S.). The authors thank Central Facility for Advanced Microscopy and Microanalysis (CFAMM) at the University of California, Riverside, for the use of SEM and

EDS and Dr. Krassimir Bozhilov for SEM/EDS training. The authors thank Dr. Lawrence D. Longo (NIH Grant HD031226) at Loma Linda University for providing sheep stifle joints for Type II collagen extraction. The authors thank Dr. Gary Botimer at Loma Linda University for the kind gift of Arithrex Osteochondral Autograft Transfer System (OATS) for harvesting the subchondral bone plugs.

Chapter 4

Adhesion of Bone Marrow-Derived Mesenchymal Stem Cells on Magnetic Poly(glycerol sebacate) Nanocomposites

4.1 Introduction

Composite is a type of material composed of more than one phase, which could be ceramic, metal or polymer. Nanocomposite is a special group of composite material that contains one or more than one phase less than 100 nm in one, two or three dimensions (DOI: 10.2147/OPENOUTLOOK.IJN.VIDEO.6). Nanocomposite materials possess different structural, mechanical, electrical, optical, electrochemical and biological properties than those of each individual component [125], they are also able to provide micro- and nano-environments to better mimic extracellular matrix when compared to traditional single or multiple phase materials [126], which makes them unique for specific applications. Various

nanocomposites have been developed for biomedical applications such as the incorporation of zinc oxide nanoparticles [127], hydroxyapatite and TiO_2 nanoparticles [128, 129] and graphene nanoplatelet [130] into different substrates, etc. Recently, with the research development on magnetic nanoparticles (MNPs), magnetic nanocomposite has drawn more and more attention in biomedical applications. Kanczler et al. showed that MNPs can be used to stimulate the differentiation of human bone marrow stromal cells toward osteogenic and chondrogenic lineages [131]. Besides, MNPs are able to respond to external magnetic field and possesses the property of superparamagnetism when they have a size of less than 20 nm [17], which offers many possibilities in biomedical applications, such as the separation of cells and bacteria [132], magnetic hyperthermia treatment of tumor cells [133], and target drug delivery [134], etc.

Poly(glycerol sebacate) (PGS) is an elastomer synthesized by Wang et al. [135], and the two reactants, glycerol and sebacic acid are approved by FDA for biomedical applications [135, 136, 137, 138]. In addition, surface degradation mechanism of PGS benefited the optimization of drug delivery applications when compared to traditional bulk erosion materials such as poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL) [139]. As a result, PGS has been extensively explored for different tissue engineering applications such as myocardial tissue [140], vascular graft [141], heart valve [142], cartilage tissue [143], contact guidance [144], and drug delivery [145] etc. However, to our best knowledge, there are currently no reports on the investigation of incorporating MNPs into PGS substrate for bone tissue regeneration.

In this study, we explored magnetic PGS nanocomposite for bone tissue engineering applications. The objective of this study was to synthesize magnetic PGS nanocomposites incorporated with different weight percent of MNPs, and determine the morphology and adhesion efficiency of bone marrow derived mesenchymal stem cells (BMSCs) on these magnetic nanocomposite scaffolds.

4.2 Materials and Methods

4.2.1 Synthesis and Characterization of magnetic nanocomposites

Synthesis of Magnetic Nanocomposites

Magnetic nanocomposites were synthesized by mixing PGS solution with different weight percent of magnetic nanoparticles and processed to flat disk-shaped samples under vacuum and high temperature. Briefly, PGS resin (RegenerezTM RG-100, Secant Medical, Inc., Telford, PA) was weighed and placed in microcentrifuge tubes, and 100% ethanol was added to the microcentrifuge tubes to dissolve PGS resin to obtain a concentration of 1g/mL. To facilitate the dissolving process, the microcentrifuge tubes were placed in a pre-heated Incu-Shaker (Model: Incu-Shaker Mini, Benchmark Scientific, Edison, NJ) at 50 °C and 160 rpm for 20 minutes. After PGS resin was completely dissolved, different amount of 1% Polyvinylpyrrolidone (PVP)-coated Fe₃O₄ nanoparticles (MNPs; #2650TR, 20 nm, Nanostructured and Amorphous Materials, Inc.) was added to PGS solution to prepare nanocomposites with 0, 0.5, 1, 5, 10 wt% of MNPs. The magnetic nanocomposites were referred by the weight percent of MNPs incorporated in it, i.e., PGS with 0, 0.5, 1, 5 and

10 wt% of MNPs incorporation was referred to as 0%, 0.5%, 1%, 5% and 10%, respectively. PGS with 0 wt% MNPs incorporation was grouped as PGS control. High-power sonicator (Model: Q125, Qsonica, LLC, Newtown, CT) combined with vortex (Model #945300, Henry Troemner, LLC, VWR, Radnor, PA) was used to disperse MNPs in PGS solution. The optimized amplitude, energy and time frame for dispersing MNPs with high-power sonicator was 90%, 14 watts and 10 minutes with 5 seconds on and 5 seconds off, respectively, under the protection of ice to prevent from heating. Every 100 μ L of PGS/MNPs suspension was pipetted out and dropped on top of Perfluoroalkoxy alkane (PFA) mold (#62670-052, VWR, Radnor, PA) for cure. The nanocomposites together with the PFA mold were placed into a vacuum oven (#3606DB, Thermo Fisher Scientific Inc., Waltham, MA) and kept at 10 torr absolute pressure and room temperature for 1 hour. After ethanol residue was removed, the temperature of the vacuum oven was increased to 120 °C for 24 hours. Then an aluminum plate (weigh 1.5 kg) was placed on top of the magnetic nanocomposites to enforce a flat-shape. Magnetic nanocomposites were then cured under 10 torr absolute pressure and 120 °C for another 24 hours.

Characterizations of MNPs and Magnetic Nanocomposites

The surface morphology of MNPs was examined using a scanning electron microscope (SEM; Nova NanoSEM450, FEI, Hillsboro, OR). An acceleration voltage of 10 kV and Everhart-Thornley secondary electron detector was used for SEM imaging. The crystal structure of the MNPs was examined using X-ray diffraction (XRD; Model: Empyrean,

PANalytical B.V., Boulder, CO) at 45kV and 40 mA with a step size of 0.026° . The XRD pattern was then analyzed using HighScore. The thickness of the PVP coating of MNPs was examined using a transmission electron microscope (TEM; Model: Tecnai12, FEI, Hillsboro, OR). A LaB_6 electron source and acceleration voltage of 120 kV was used.

Magnetic PGS nanocomposites were characterized with a digital Canon SLR camera (Model: EOS Rebel T3i Digital Canon SLR, Canon USA, Inc., Melville, NY) and SEM with spot size 3 and acceleration voltage of 3 kV. The magnification of $10,000\times$ was reached to detect the surface features of magnetic nanocomposites. XRD was used to determine the presence of MNPs in magnetic nanocomposites.

4.2.2 Response of Magnetic Nanocomposites to External Magnetic Field

The response of magnetic nanocomposites to external magnetic field before cell study was tested using a N42 neodymium magnet (#DEX0, K&J Magnetics, Inc., Pipersville, PA) with a diameter of 22 mm and a height of 25 mm. The magnet was embedded in the testing machine named 3D magnetic controller, which was built in our lab. The 3D magnetic controller was composed of a wood platform, a battery case, a motor, a digital display LED, a switch and a speed controller. The nanocomposites were able to be free-floating on DI water filled in a petri dish with diameter of 8 cm due to surface tension. The bottom surface of the magnet was placed 2.5 cm higher than the top surface of the magnetic nanocomposites and moved in a speed that could direct the movement of nanocomposites in a circular motion without delay. The circle diameters and time used to complete

the whole circle for different group of magnetic nanocomposites were recorded. In another study, the nanocomposites were moved from one side of the dish to the other side, and the responding time was recorded for all groups. The average moving speed of each group of nanocomposites was calculated and compared.

4.2.3 Mechanical testing

The mechanical properties of the magnetic PGS nanocomposites were characterized by tension and compression measurements using an Instron 5969 dual column testing system equipped with 10 N and 50 kN load cell (Instron, Norwood, MA), respectively.

Specimens were prepared as rectangular strips of size $12.5 \times 3 \times t$ mm (t = thickness) for tension test. Tension test was conducted with a 0.005 N preload and cross-head speed of 10 mm/minute until the breakage of the specimen. Three samples were tested of each group of nanocomposites and pure PGS ($n = 3$). The tensile stress-strain curves were plotted from the calculation of the load-extension data. The stress was the load divided by the cross-section area of specimens and the strain was the extension divided by the gauge length of the specimens. The elastic Youngs modulus was determined from the linear region of stress-strain curve by fitting a straight line between 0% and 20% strain. Percent elongation until breakage of samples were calculated.

Specimens were also prepared as disks with diameter of 6 mm and thickness of t mm for compression test. Compression took place with a 0.05 N preload and cross-head speed of 10 mm/minute until the load reached 5 kN, which allowed the crush of the

specimens. Three samples were tested of each group of nanocomposites and pure PGS ($n = 3$). The compression stress-strain curves were plotted similarly as tension stress-strain curves. The Youngs modulus of compression was defined as the gradient of the stress-strain curve in 0% to 20% strain region.

4.2.4 *In Vitro* Cytocompatibility Study with BMSCs

Sample Preparation for Cell Culture

To determine the necessity of conducting pretreatment of magnetic nanocomposites before cell study, we separated magnetic nanocomposites into two groups, in which a group was pretreated while the other group was not. Both groups included triplicate 0, 0.5, 1, 5 and 10% samples. The pretreatment process was modified from Wang et al. [146]. Briefly, for the group with pretreatment, all the samples were soaked serially in 70, 50 and 25% ethanol for 1 hour each, then ethanol was removed and followed by soaking twice in phosphate buffered saline (PBS; #2810305, MP Biomedicals, LLC, Solon, OH) for 1 hour each. Afterwards, the samples were conditioned in complete Dulbeccos modified Eagles medium (cDMEM), which contains Dulbeccos modified Eagles medium (DMEM; #15-013-CM, Mediatech, Inc., Manassas, VA) and supplemented with 10% fetal bovine serum (FBS; #SH30910, HyClone, Thermo Fisher Scientific Inc., Waltham, MA) and 1% Penicillin/Streptomycin (P/S; #SV30010, HyClone, Thermo Fisher Scientific Inc., Waltham, MA), for 24 hours in standard cell culture environment (i.e., 37 °C, 5% CO₂/95% air, humidified environment) before transferred to 24-well cell culture plate.

For the group without pretreatment, all the samples were sterilized with 70% ethanol for 30 minutes, then ethanol was removed and samples were allowed to dry for 10 minutes and moved to another 24-well plate for cell study.

MNPs were placed in a microcentrifuge tube and exposed to ultraviolet radiation for 1 hour to disinfect. After sterilization, MNPs of 36 mg were suspended in 1.2 mL of cDMEM homogeneously. Each 200 μ L of the MNPs suspension was gently pipetted into each of the three wells of two 24-well plates. cDMEM in each well was then gently pipetted out without disturbing the MNPs.

***In Vitro* Cytocompatibility Study with BMSCs**

A confluent flask of bone marrow-derived mesenchymal stem cells (BMSCs) was washed with 10 mL PBS, detached by adding 3 mL trypsin (#25200-056, Life Technologies, Carlsbad, CA) and incubated under standard cell culture conditions for 3 minutes. 9 mL cDMEM was added to the flask after 3 minutes to neutralize trypsin and the cell suspension was collected into a 15 mL-centrifuge tube and centrifuged for 5 minutes at 1000 rpm. The supernatant was aspirated and 5 mL fresh cDMEM was added into the tube to resuspend the cells. 10 μ L cell suspension was dropped onto a small petri dish and then 10 μ L of trypan blue solution (#T8154, Sigma-Aldrich, St. Louis, MO) was mixed with the cell suspension. The mixture was loaded into the hemocytometer (#1475, Reichert Bright-Line, Hauser Scientific, Horsham, PA) and cells were counted manually under microscope. Cell suspension volume needed for seeding (10,000 cells/cm²) was calculated and diluted with

cDMEM as needed. 2 mL of cell suspension (at a seeding density of 10,000 cells/cm²) was pipetted into all study wells of the two 24-well plates, including thirty wells with pretreated or non-pretreated magnetic nanocomposites, six wells of the MNPs group with 6 mg of MNPs in each, and six wells of the control group with just cells and cDMEM. The plates were gently placed back into the incubator and cultured for 24 hours under the standard cell culture conditions.

After 24 hours, all the cDMEM were removed from each well and media pH was tested. Non-adherent cells were removed by washing with PBS. Adherent cells on the 24-well plate or on the samples were both fixed with 4% paraformaldehyde (PFA; #15714-S, Electron Microscopy Science, Hatfield, PA) solution, stained with Alexa Fluor 488 (#A12379, Life Technologies, Carlsbad, CA) for F-actin and 4',6-diamidino-2-phenylindole (DAPI; #D3571, Life Technologies, Carlsbad, CA) for cell nuclei. After fixing and staining, cells were imaged using a fluorescence microscope (Nikon Ti-S eclipse, Nikon, Japan) and the images were merged using ImageJ, and cell density in each well was calculated as well. Briefly, five spots in each well were randomly selected for imaging and cell numbers in each image were manually counted. Average cell density in each well was calculated.

4.2.5 Statistical Analysis

Experiments were run in triplicate and the data were analyzed in R using one-way analysis of variance (ANOVA) followed by the Tukey highest significant difference post hoc test. Statistical significant difference was considered at $p < 0.05$.

4.3 Results

4.3.1 Characterization of MNPs and Magnetic Nanocomposites

SEM image at a magnification of $50,000\times$ showed the sphere shape of the MNPs (Figure 4.1A) and XRD spectrum showed the presence of the major peaks of Fe_3O_4 , which were at 30.27° , 35.65° , 43.27° , 53.69° , 57.24° and 62.86° , and those peak positions were highly consistent with the standard XRD pattern of Fe_3O_4 (JCPDS #65-3107), indicating the nanoparticles are in Fe_3O_4 phase (Figure 4.1B). TEM image in Figure 4.1C showed the thickness of the PVP coating was 1.5 nm and the MNPs were in a sphere shape, which was consistent with the result of SEM examination. The lattice spacing of MNPs was measured to be 0.488 nm, which was indexed to the (111) plane of Fe_3O_4 crystal structure (Figure 4.1D) [73].

Optical images of magnetic PGS nanocomposites with different weight percent of MNPs showed a color change. PGS control exhibited a translucent color, while with the addition of MNPs ratio from 0.5 wt% to 10 wt%, the color of PGS nanocomposites ranged from light brown to completely black (Figure 4.2). All the samples exhibited a round shape and had a diameter around 7 mm. SEM analysis showed that there was no apparent surface feature on magnetic PGS nanocomposites at the magnification of $10,000\times$ except the presence of random MNPs agglomerates, and the whole structure is non-porous (Figure 4.2A-E). The XRD spectrum of pure PGS control showed a big, wide peak that indicated the amorphous structure of PGS. While for the magnetic PGS nanocomposites, peak intensities

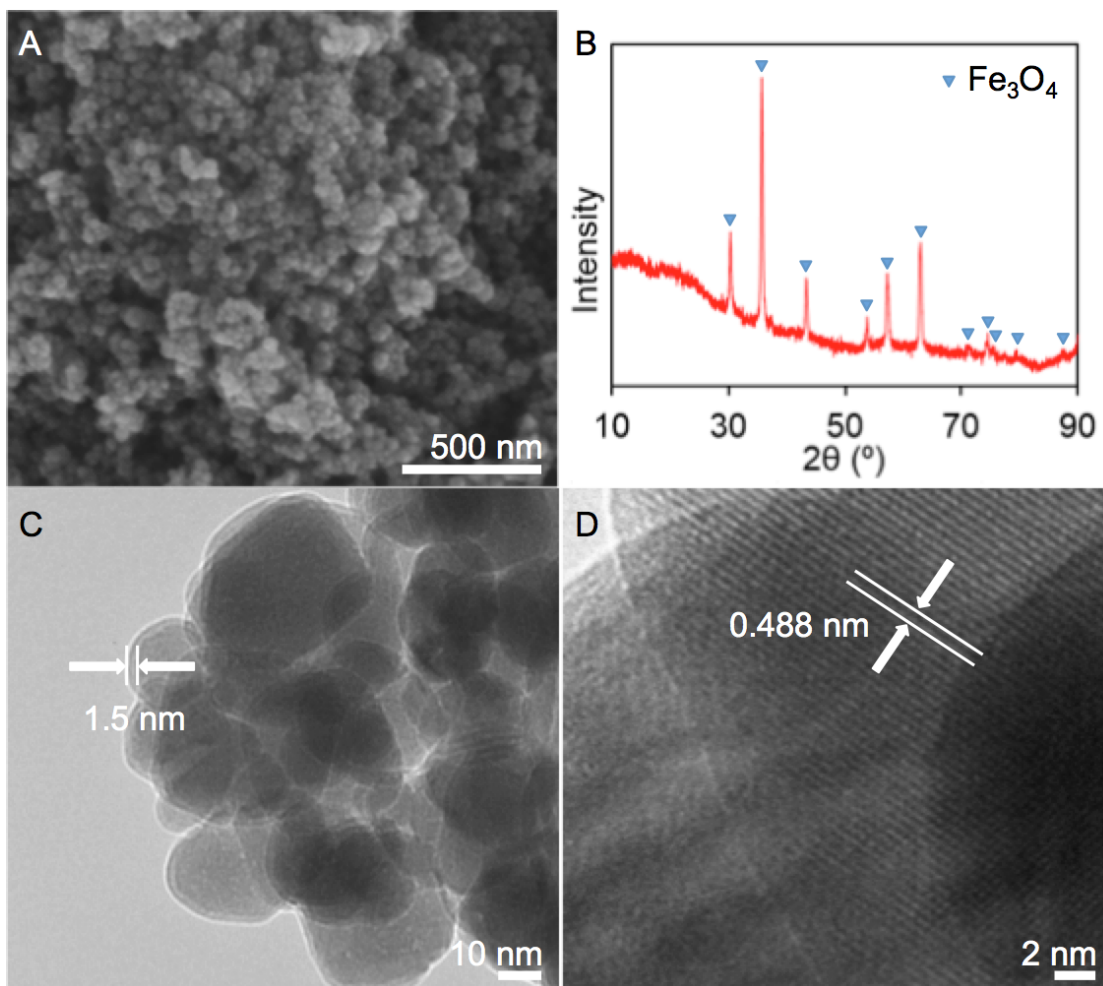


Figure 4.1: Characterization of the MNPs used in the magnetic nanocomposites. (A) SEM image of the MNPs (1% PVP-coated Fe_3O_4 nanoparticles). Original Magnification = 50,000 $\times$, scale bar = 500 nm. (B) X-ray diffraction pattern of the MNPs. Step size = 0.026°. Fe_3O_4 standard pattern PDF #19629. (C) TEM image of the MNPs. The PVP coating was measured to be 1.5 nm. Original magnification = 150,000 $\times$, scale bar = 10 nm. (D) High-magnification TEM image showing the lattice planes. The interplanar distance was measured to be 0.488 nm, which is indexed to the (111) plane of Fe_3O_4 crystal structure. Original Magnification = 740,000 $\times$, scale bar = 2 nm.

increased when the weight percent of MNPs in magnetic PGS nanocomposites increased (Figure 4.2F).

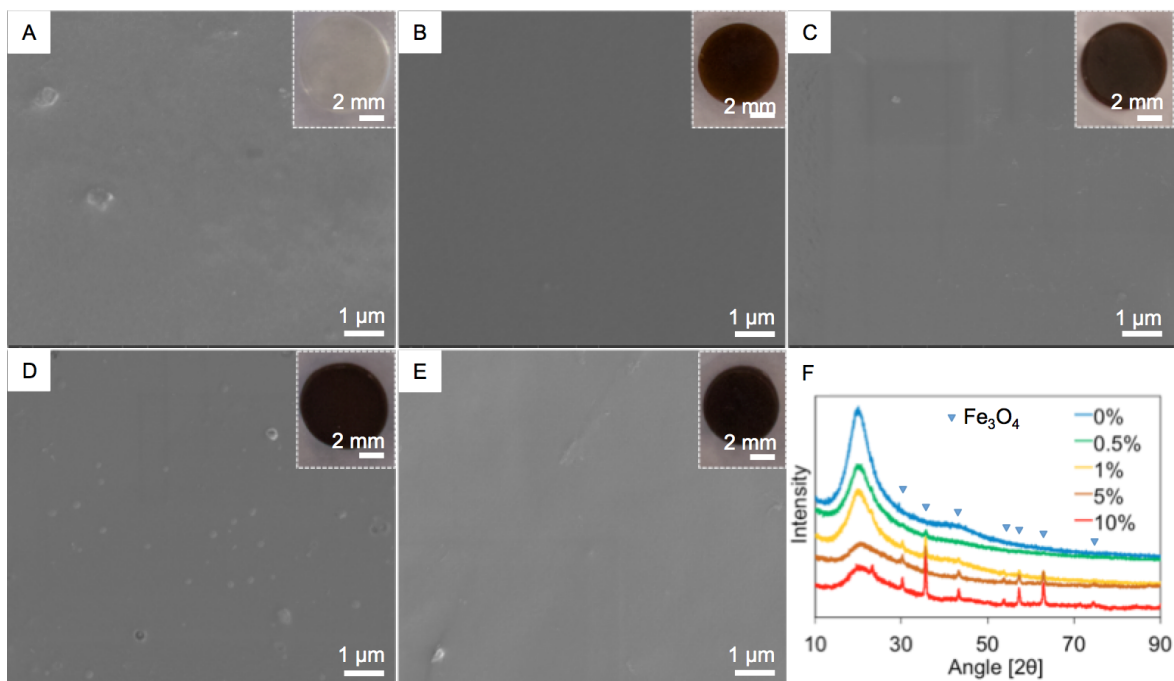


Figure 4.2: Characterization of magnetic nanocomposites with increasing weight percent of MNPs. Each type of magnetic nanocomposite was referred by the weight percent of MNPs incorporated in it. SEM images of (A) PGS/0%MNPs (0%), (B) PGS/0.5%MNPs (0.5%), (C) PGS/1%MNPs (1%), (D) PGS/5%MNPs (5%) and (E) PGS/10%MNPs (10%). Original magnification = 10,000 $\times$. Scale bar = 1 μ m. Insertion: photographs of corresponding magnetic nanocomposites. Scale bar = 2 mm. (F) X-ray diffraction pattern of the magnetic nanocomposites. Step size = 0.026 $^\circ$, Fe₃O₄ standard pattern JCPDS #65-3107.

4.3.2 Response of Magnetic Nanocomposites to External Magnetic Field

The schematic diagram of the 3D magnetic controller and a photograph of top control unit was shown in Figure 4.3A. Figure 4.3B showed that magnetic nanocomposite with 0.5 wt% of MNPs incorporation had an average speed of 1.82 ± 0.07 and 1.54 ± 0.30 cm/s in circular motion and linear motion, respectively. And with the increase of MNPs

incorporation, the moving speed of magnetic nanocomposite in the circular/linear motion test increased to $2.93 \pm 0.18/3.38 \pm 0.27$ and $12.94 \pm 1.55/9.32 \pm 0.96$ cm/s for 1% and 5%, respectively. However, the moving speed of the 10% was 11.77 ± 0.60 and 10.82 ± 1.71 cm/s in the circular motion test and linear motion test, respectively, which kept the same when compared with 5%. Moving speed tested in linear motion was similar to the results tested in circular motion. The moving speed of 5% and 10% was significantly higher than the moving speed of 0.5% and 1% magnetic nanocomposites (see supplementary video).

4.3.3 Mechanical testing

Youngs modulus and percent elongation were calculated from the stress-strain curves and illustrated in Figure 4.4. From test results, the tensile Youngs modulus of pure PGS is 0.259 ± 0.024 MPa and PGS can be elongated to at least two times of its original length without rupture. The values presented here are mean $\pm$ standard error of mean. The results are consistent with the work published by Wang et al. by 2002 [135]. The addition of MNPs to pure PGS resulted in changes in tensile modulus and elongation compared to pure PGS network. The addition of 0.5% MNPs resulted in small increase in Youngs modulus and minor decrease in elongation, while when the addition of MNPs was 1%, the Youngs modulus decreased and elongation increased. The Youngs modulus kept increasing from 5% to 10% but elongation decreased and increased for 5% and 10% groups, respectively. The Youngs modulus of 10% group reached 0.372 ± 0.025 MPa, which was significantly higher than 1% group. The ultimate tensile strength of 0% and 10% group was similar,

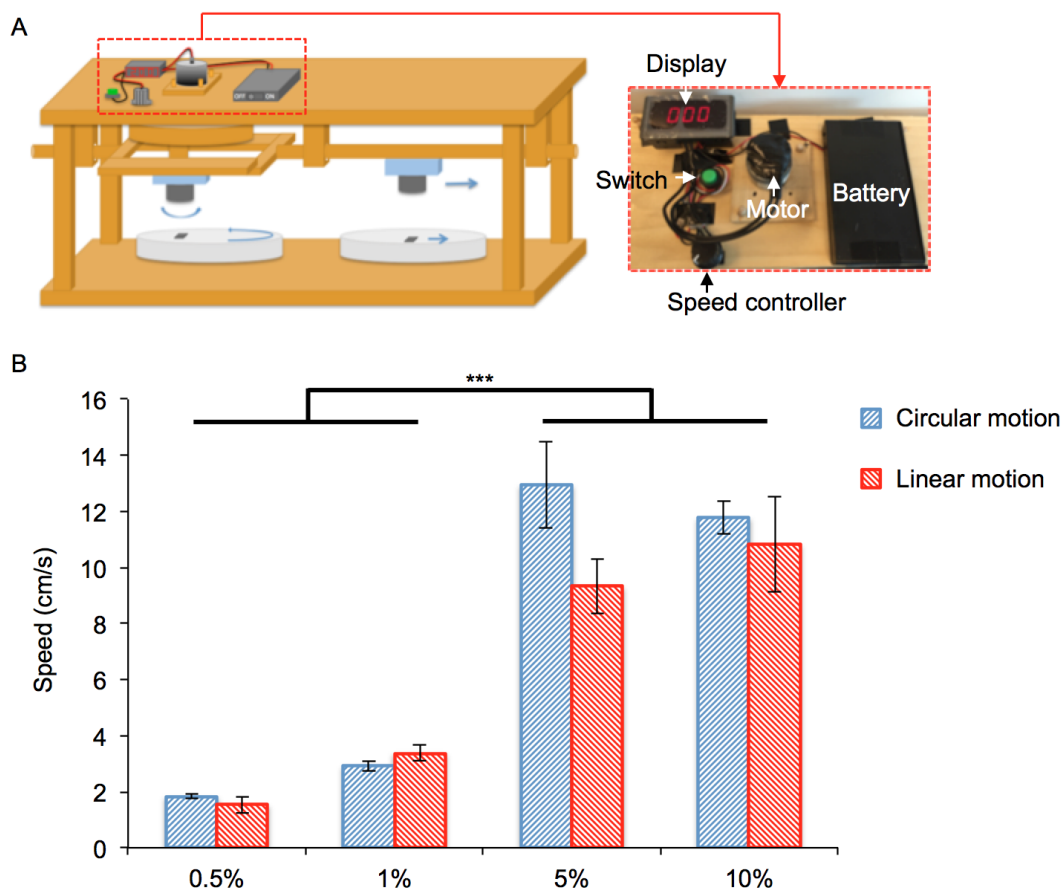


Figure 4.3: Remote guidance of the magnetic nanocomposites under a tunable speed using a 3D magnetic controller. Videos are available in supplementary materials. (A) The schematic design of the 3D magnetic controller (left) and the photograph of the control unit (right). (B) The speed of magnetic response for each group of magnetic nanocomposites. The response speed of 5% and 10% are significantly higher than 0.5% and 1% in both circular motion test and linear motion test. The speed increased with increasing weight percent of MNPs from 0.5% to 5%. There were no significant difference in response speed of 5% and 10%. Values are mean $\pm$ standard deviation, $n = 3$; *** $p < 0.001$.

and higher than the 0.5, 1, and 5% groups, however, there was no significant difference among all groups. The increase in Young's modulus always accompanied with decrease in elongation, however, this did not apply to the 10% magnetic PGS nanocomposite group, which had similar elongation as pure PGS and higher Young's modulus.

The relative relationship of compressive modulus of the five groups are similar to elastic Young's modulus but the values increased with a magnitude (Figure 4.5). The test result of pure PGS is consistent with previous publications [147]. The compressive modulus of pure PGS, 0.5%, 1%, 5% and 10% groups were 2.096 ± 0.168 MPa, 2.517 ± 0.314 MPa, 1.710 ± 0.110 MPa, 1.753 ± 0.057 MPa, and 2.364 ± 0.116 MPa. However, there was no significant difference in compressive modulus among those groups. The samples were broken and stickier after test, showing that the compressive stress induced the breakage of weak bondings and high-pressure might partially reversed the curing of PGS.

4.3.4 *In Vitro* Cytocompatibility Study with BMSCs

Cells Morphology on the Samples

There were a few BMSCs presented on the surface of the magnetic nanocomposites (direct contact) in the group with pretreatment, and the shape of cells was normal when compared with cell only group (Figure 4.6). While in the group without pretreatment, there were barely healthy cells presented on the magnetic nanocomposites and the presence of cell debris indicated cell apoptosis. Moreover, it was unable to distinguish cell nuclei (DAPI

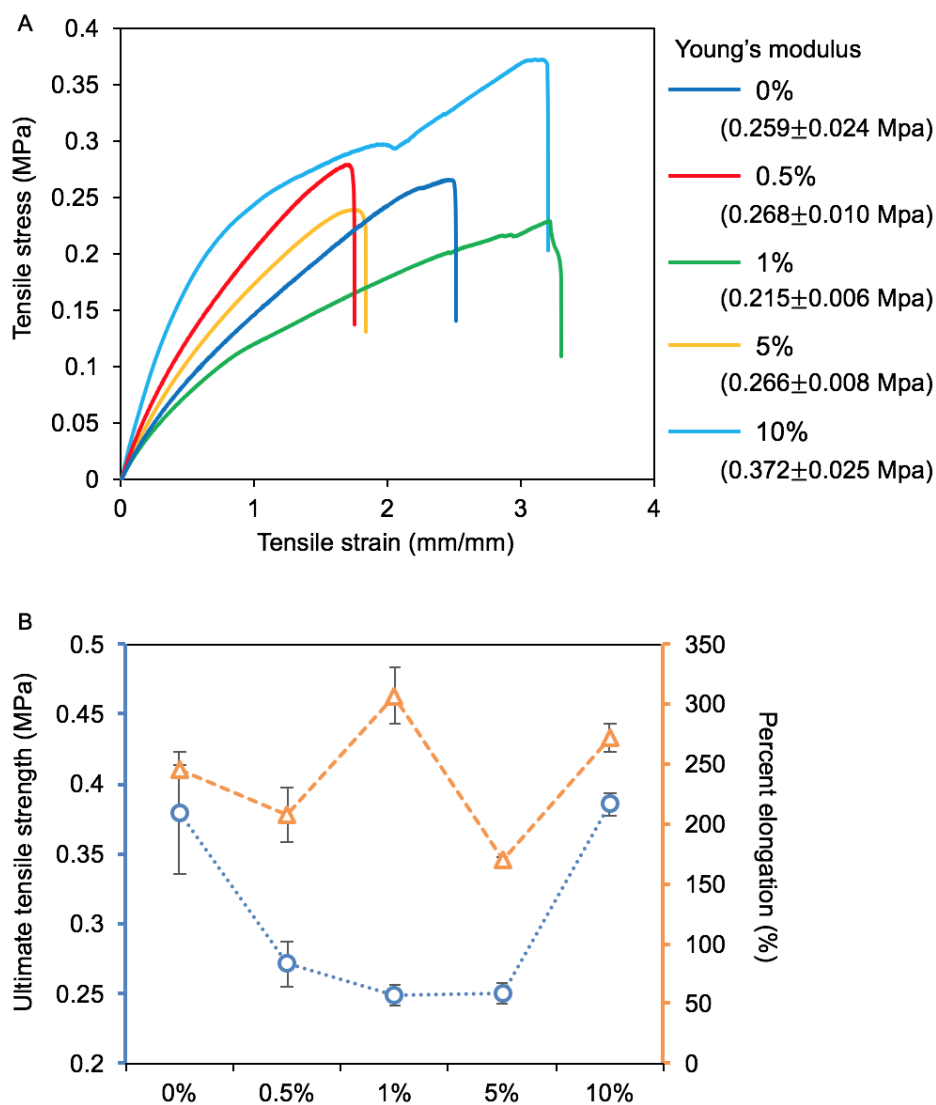


Figure 4.4: Tension test of magnetic PGS nanocomposites. (A) Tensile stress-strain curves for 0, 0.5, 1, 5 and 10% MNPs-incorporated 2D non-porous PGS nanocomposites with Young's modulus labeled on the right. (B) Ultimate tensile strength and percent elongation of 0, 0.5, 1, 5 and 10% MNPs-incorporated 2D non-porous PGS nanocomposites. Values are means $\pm$ standard error of mean, $n = 3$.

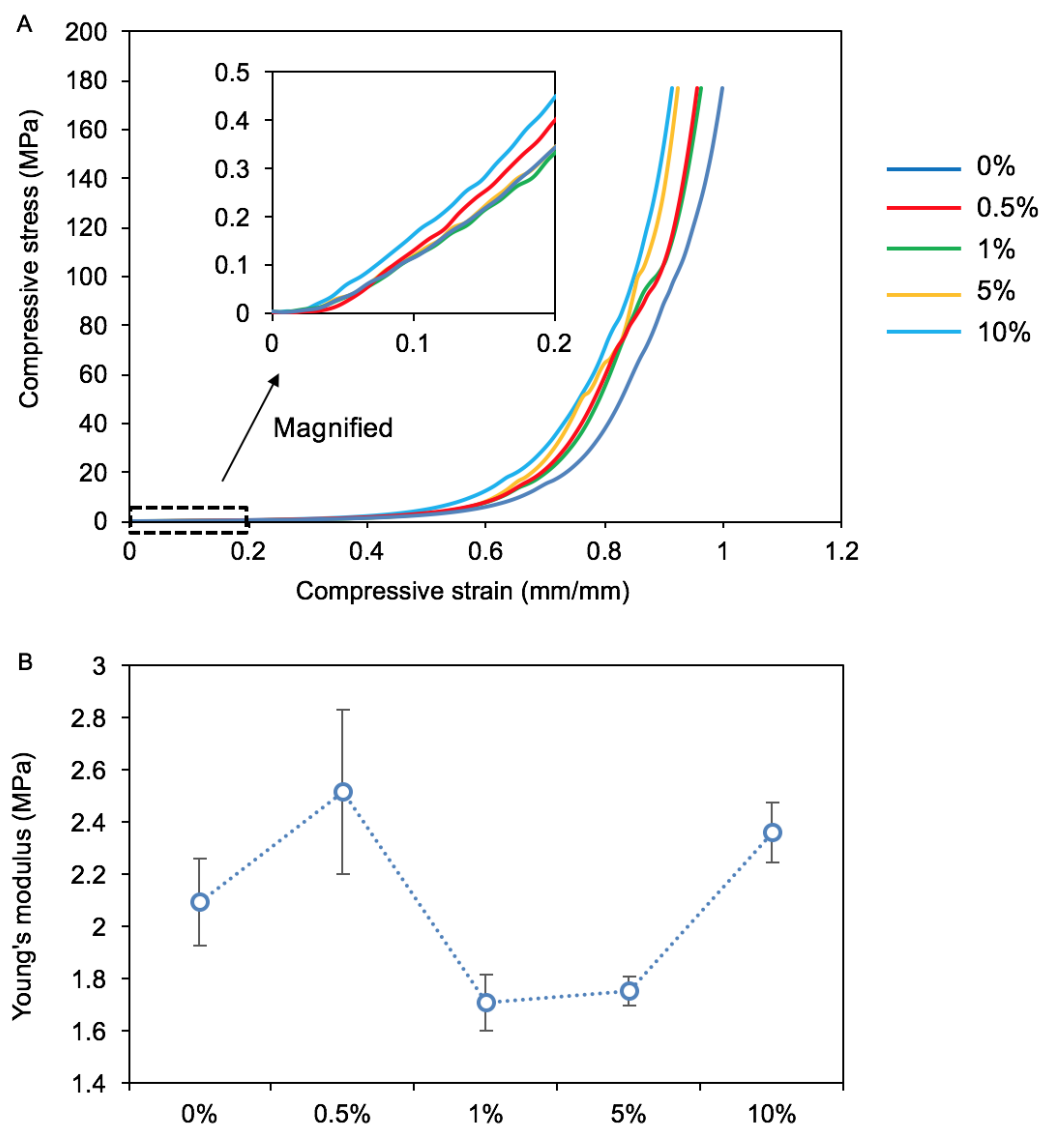


Figure 4.5: Compression test of magnetic PGS nanocomposites. (A) Compressive stress-strain curves for 0, 0.5, 1, 5 and 10% MNPs-incorporated 2D non-porous PGS nanocomposites. (B) Young's modulus of 0, 0.5, 1, 5 and 10% MNPs-incorporated 2D non-porous PGS nanocomposites.

staining), and cell F-actin was faded when compared to the cells grown on the plate in the same well.

Cells Morphology on the Plate

Morphology of BMSCs in the group with pretreatment, whether on the plate (indirect contact) close to the magnetic nanocomposites (area a) or away from the magnetic nanocomposite (area b), were all in normal healthy shapes when compared with cell only group (Figure 4.6). While BMSCs close to magnetic nanocomposites (area a) in the group without pretreatment were more star-shaped and had unusual thin branches pointing from the main cell body to any directions. The cells nuclei were also changed from healthy oval shape to horse hoof shape or unusually bright when stained with DAPI and Alexa Flour 488. The change in overall cell shape indicated cell apoptosis.

Cell Density on the Samples

Only a few cells were found in the group with pretreatment and the group without pretreatment, and the cell densities on the magnetic nanocomposites (direct contact) in the group with pretreatment were higher than cell densities in the group without pretreatment (Figure 4.7A). However, the cell density on magnetic nanocomposites was significantly lower when compared to initial cell seeding density (10,000 cells/cm²).

The overall cell densities on the plate in each pretreated group were all higher than in its corresponding non-pretreated group. The overall cell density on the plate in

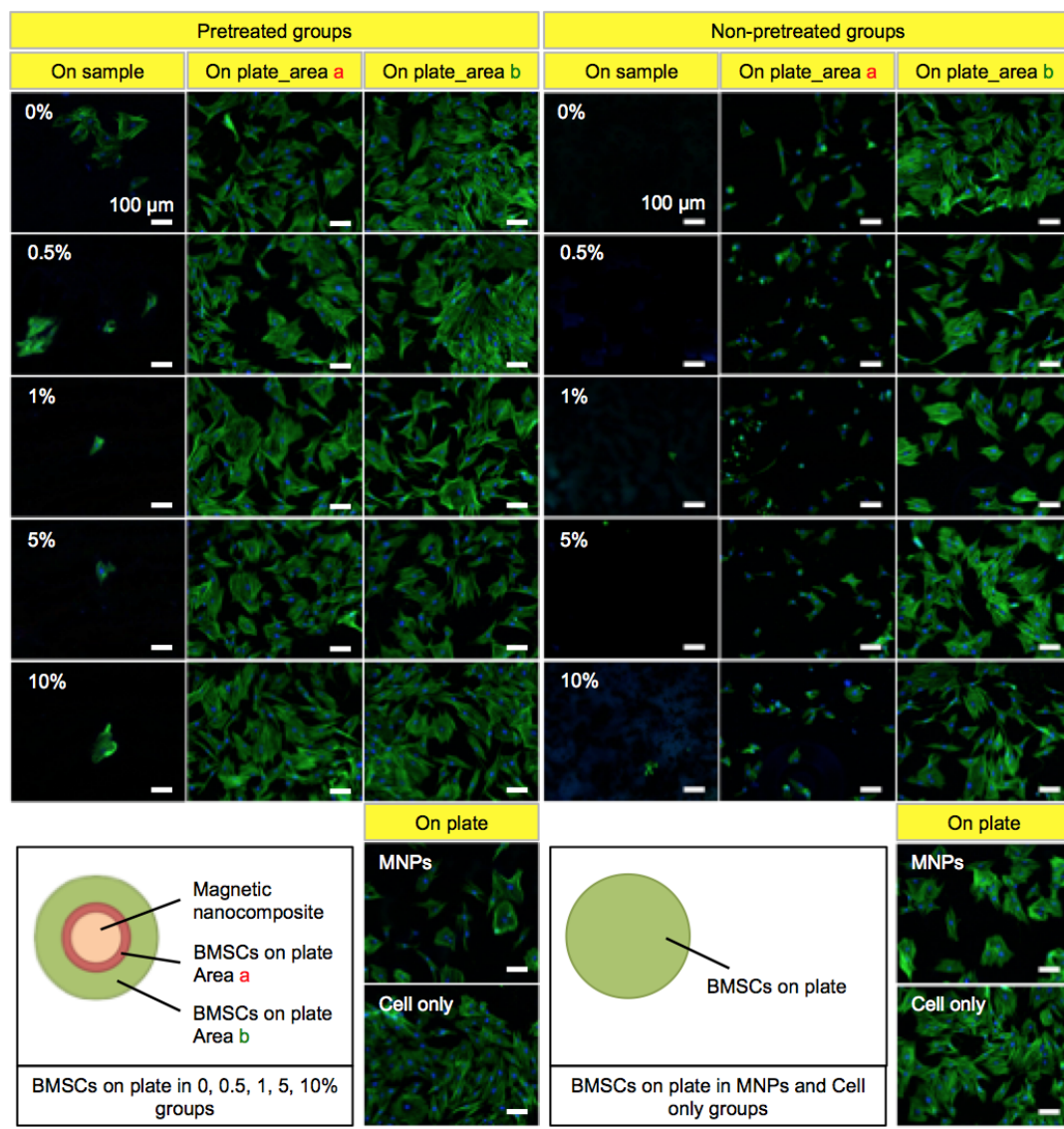


Figure 4.6: Fluorescence images of BMSCs after 24 hours of culture with magnetic nanocomposites with increasing weight percent of MNPs. BMSCs attached directly on the samples, i.e., direct contact with samples, and attached on the culture plates surrounding the samples, i.e., indirect contact with samples, were imaged separately. The pretreated nanocomposite groups were compared with those not. BMSCs attached on the plates near the 0, 0.5, 1, 5, 10% samples (area a) and away from the samples (area b) were compared. BMSCs were distributed homogeneously in the MNP's groups and cell only groups, which contained no separate areas. Scale bar = 100 μm .

the pretreated group had an increasing trend from 0% group to 10% group (Figure 4.7B). BMSCs cultured with MNPs in the group with or without pretreatment were both had a lower cell density than other groups (Figure 4.7B), which could be caused by the removal of MNPs during staining process if there were cells attached to the MNPs.

In the groups with both pretreated and non-pretreated magnetic nanocomposite samples, cell distribution was non-homogeneous when compare the area close to the samples with the area away from the samples, and consequently, a further analysis on cell distribution was conducted. The results showed that cell densities on the plate close to samples (area a) in the group with pretreatment were all higher than the densities on the plate close to samples in the group without pretreatment, but the cell densities in each group were all lower than the initial seeding density (Figure 4.7C). Cell densities on the plate away from the samples (area b) in the group with pretreatment were all significantly higher than the cell densities on the plate away from the samples in the group without pretreatment. And cell densities on the plate away from the samples in the group with pretreatment were all higher than the seeding density while cell densities on the plate away from the samples in the group without pretreatment still lower than the initial seeding density (Figure 4.7D).

Change of Media pH

For the group with pretreatment, the pH of the media in each well after 24 hours of culture was centered at 8.2 and ranged from 8.1 to 8.4. For the group without pretreatment, the pH was centered at 7.7 and ranged from 7.6 to 7.9, which was significantly lower than

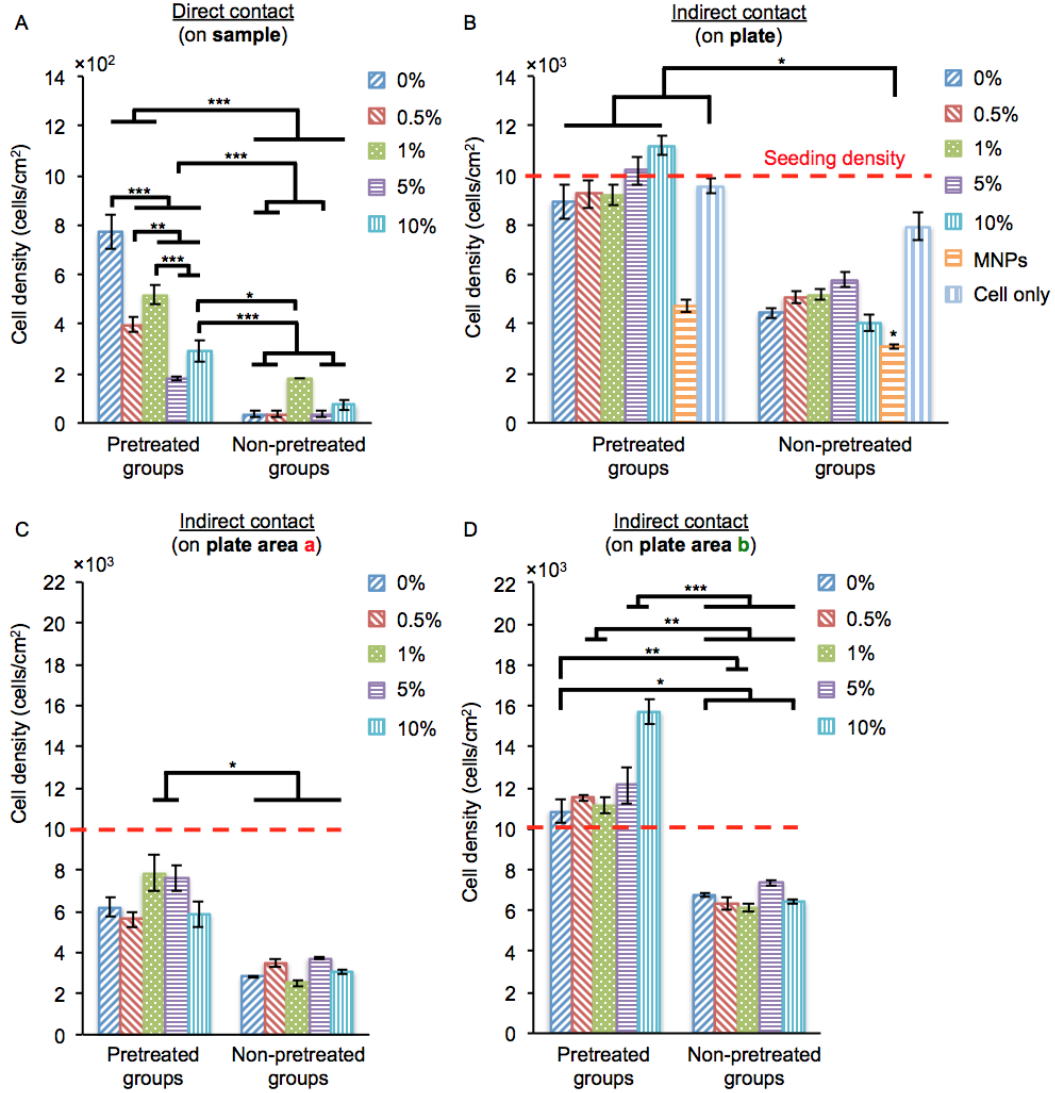


Figure 4.7: Quantified densities of BMSCs after 24 hours of culture with magnetic nanocomposites with increasing weight percent of MNPs. (A) BMSCs adhered on the samples (direct contact with samples), (B) overall BMSCs density on the plates in each group (indirect contact with samples), (C) BMSCs adhered on the plates near the samples (indirect contact with samples, area a) and (D) BMSCs adhered on the plates away from the samples (indirect contact with samples, area b). The pretreated nanocomposite groups showed greater cell densities than the non-pretreated groups in average. Red dashed lines indicated the initial BMSCs seeding density. Values are mean $\pm$ standard error of means, $n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

the group with pretreatment (Figure 4.8). There was no statistically significant difference of pH within each group.

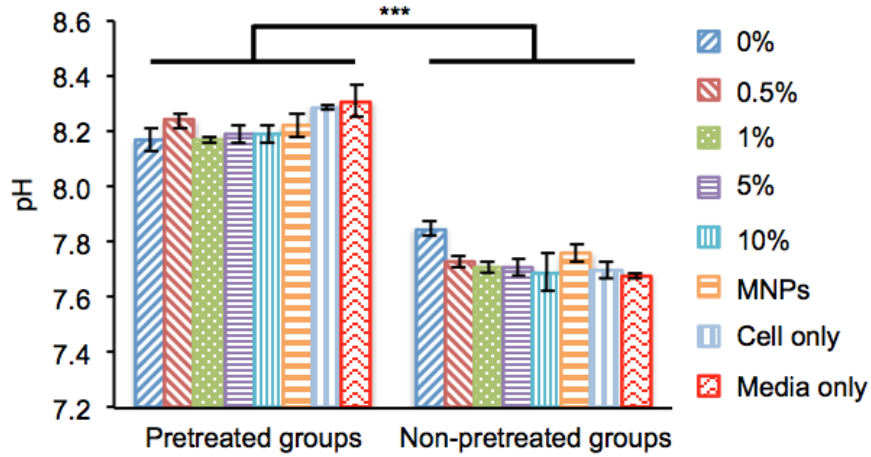


Figure 4.8: Media pH after 24 hours of cell culture with magnetic nanocomposites with increasing weight percent of MNPs. The nanocomposites without pretreatment showed statistically lower pH in post-culture media than those with pretreatment. Values are mean standard error of means, $n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.4 Discussion

According to the results of tension and compression tests, these magnetic PGS nanocomposites demonstrated similar mechanical properties as the pure PGS. Tension tests of magnetic PGS nanocomposites have shown that the materials exhibit nonlinear stress-strain behavior, which is typical for soft elastic materials [148]. And the stress-strain curves of magnetic PGS nanocomposites and pure PGS are similar to what have been reported by other researchers [142, 149, 150]. The elastic property of PGS is due to the covalent

crosslinking and hydrogen bonding interactions of hydroxyl groups attached to its backbone [148].

It is speculated that, the hydroxyl groups on the surface of MNPs have crosslinked with the hydroxyl groups on the backbone of the polymer, which may affect the mechanical properties of the magnetic PGS nanocomposite. Nevertheless, the Youngs modulus both from the tensile and compressive test presented similar results among all groups, and the percent elongation of all groups are in the same range. In comparison to natural tissue, the Youngs moduli of the magnetic PGS nanocomposites are close to cartilage (0.7-15.3 MPa) and myocardium (0.02-0.5 MPa), while compressive moduli were closer to low-density cancellous bone (about 10 MPa) [151]. The mechanical properties of magnetic PGS nanocomposite made it good candidate for soft tissue repair and its magnetic property enables remote magnetic manipulation of implanted scaffolds.

The in vitro study observed fewer cells on the magnetic nanocomposites than on the plate in both the group with and without pretreatment. It was clearly shown in the SEM micrograph (Figure 4.2A-E) that the surface of magnetic nanocomposites exhibited smooth character without obvious micro- or nano-sized structures, which could potentially decrease the efficiency of cell attachment. Besides, the magnetic nanocomposites degraded through surface-erosion mechanism during the 24 hours of cell study [139], and consequently, the cells initially attached on the surface layer of the nanocomposites were detached together with the degradation of surface layers.

Cell densities on the plate in both the MNPs wells and cell only wells should be the same in both the pretreated groups and non-pretreated groups due to the same seeding density and same culture environment. Although the cell densities on the plate in the MNPs wells and cell only wells in the non-pretreated group were lower than them in the pretreated group, there was no significant difference, showing the consistency of cell seeding.

There was more healthy cells indirect contact with magnetic nanocomposites in the group with pretreatment when compared to the group without pretreatment. The media pH in the group with and without pretreatment was all relatively similar within the same group, but the pH in the group with pretreatment was significantly higher than it in the group without pretreatment. In the group with pretreatment, the unreacted monomers glycerol and sebacic acid were removed in the series soaking process before cell study. We hypothesized that without the pretreatment, the unreacted monomers would be leached out to the DMEM environment during the in vitro cell culture process, resulted in a relatively acidic environment and caused damage to the cells. This could also be one of the reasons that resulted in the lower cell density on the magnetic nanocomposites when compared to cell density on the plate.

4.5 Conclusions

Unreacted monomers resided in the magnetic nanocomposites after curing resulted in a relatively acidic environment during direct contact with cells and caused cell death. A series soaking pretreatment process helped remove the unreacted monomers in magnetic

nanocomposites before direct contact with BMSCs. The mechanical properties of magnetic PGS nanocomposite made it ideal candidate for soft tissue engineering, however, the smooth surface and surface degradation mechanism of magnetic nanocomposites are not ideal for cell attachment. An investigation on the porous magnetic nanocomposites will be conducted to increase cell adhesion. The magnetic response speed of the nanocomposites with different ratio of MNPs incorporation is beneficial for the determination of MNPs percentage in future clinical magnetic implants.

4.6 Acknowledgements

We would like to thank Central Facility for Advanced Microscopy (CFAMM) at the University of California, Riverside, for the use of SEM, and Ilkeun Lee and Dr. Krassimir Bozhilov for the training on SEM. We thank Dr. Mustafa Ozgul for the help with TEM.

Chapter 5

Improved Adhesion of Bone Marrow-Derived Mesenchymal Stem Cells on 3D Porous Magnetic Poly(glycerol sebacate) Nanocomposites

5.1 Introduction

The previous chapter showed that 2D non-porous PGS nanocomposites had low cell adhesion, thus, porous structure with improved surface features was brought up to increase cell adhesion efficiency. Moreover, porous structure has the benefits of increasing nutrition exchange and diffusion of waste products out of the scaffold. In this study, we synthesized 3D porous magnetic PGS nanocomposite scaffolds incorporated with different ratio of magnetic nanoparticles to compare with 2D non-porous magnetic PGS nanocom-

poistes, and determine the adhesion efficiency and morphology of bone marrow derived mesenchymal stem cells (BMSCs) on these porous magnetic nanocomposite scaffolds.

5.2 Materials and Methods

5.2.1 Synthesis and Characterization of 3D Porous Magnetic PGS Nanocomposites

Synthesis of Magnetic Nanoparticles

MNPs with 30 wt% of polyvinyl alcohol (PVA) as the surface modifier were synthesized using the co-precipitation method modified from our previous work [72]. Briefly, PVA (#341584, molecular weight 89000-98000, Sigma-Aldrich, St. Louis, MO) was dissolved in deionized water (DI water; Milli-Q Integral Water Purification System, EMD Millipore, Billerica, MA) under the temperature of 100 °C for 30 minutes to make 18.96 mg/mL PVA solution. FeCl₃ (#169430010, Sigma-Aldrich, St. Louis, MO) and FeCl₂·4H₂O (#44939, Sigma-Aldrich, St. Louis, MO) powder with a molar ratio of 2:1 was then dissolved in the PVA solution and stirred at room temperature for additional 30 minutes. A 25½ gauge needle was used to gradually drop the aqueous mixture of PVA/FeCl₃/FeCl₂·4H₂O into 50 mL of 10%-diluted ammonium hydroxide solution (#338818, Sigma-Aldrich, St. Louis, MO) to produce magnetic nanoparticles. MNPs were washed with DI water, dried in a vacuum oven under 80 °C for 3 hours, and ground using a mortar and pestle.

Characterization of Magnetic Nanoparticles

The surface morphology of MNPs was examined using a scanning electron microscope (SEM; Nova NanoSEM450, FEI). MNPs were mounted onto the SEM sample holder using the copper tape. An acceleration voltage of 10 kV and Everhart-Thornley secondary electron detector was used for SEM imaging. The size of MNPs was analyzed based on the high-resolution SEM image using the quantitative analysis tools of ImageJ software, with which 200 MNPs were measured and the average Feret diameter was calculated with an assumption that the synthesized MNPs are spherical. The crystal structure of the MNPs was analyzed using X-ray diffraction (XRD; PANalytical X-ray diffractometer, Empyrean) at 45kV and 40 mA with a 0.026° step size. The magnetization of the MNPs was tested with a magnetometer (7400 Series VSM, Lake Shore Cryotronics, Inc. Henderson, NV) in the testing field range of -10000 to 10000 gauss and a hysteresis loop was obtained for analysis.

5.2.2 Synthesis and Characterization of 3D Porous PGS Nanocomposites

Synthesis of 3D Porous PGS Nanocomposites

In our previous study, we were able to prove that a non-porous PGS nanocomposite without any surface features had a low percentage of cell attachment, so we created porous structures in the nanocomposites to increase cell adhesion efficiency. PGS resin was purchased from Secant Medical (Secant Medical, Inc. Telford, PA) and processed to porous scaffolds use salt leaching method adapted from Wang et al. [146]. Briefly, NaCl

salt crystals (Sigma) was ground and sieved to the size of 125-150 μm and added to a PFA mold with size of $8.2 \times 3.7 \times 1.8$ cm. 2 mL of 100% ethanol was added subsequently to the mold with gently shaking to achieve homogeneous dispersion of NaCl crystals in the mold. The whole setup was air dried for 5 hours to produce a flat salt template with a thickness of 200 μm . The salt template was then incubated in an incu-shaker (#H1000-M, Incu-shaker mini, Benchmark Scientific, Inc. South Plainfield, NJ) with relative humidity of 88-95% for 2-3 hours to fuse the NaCl crystals. Afterwards, it was dried in a vacuum oven under 60 $^{\circ}\text{C}$ and 10 torr absolute pressure for 12-18 hours. PGS resin was dissolved in 100% ethanol to a concentration of 513.9 mg/mL, and then different amount of MNPs was added to PGS solution for the preparation of PGS nanocomposites with 0, 0.5, 1, 5 and 10 wt% of MNPs incorporation. The PGS nanocomposites with 0, 0.5, 1, 5 and 10 wt% MNPs were referred to as 0%, 0.5%, 1%, 5% and 10%, respectively. High-power sonicator combined with vortex was used to disperse MNPs in PGS solution. The optimized amplitude, energy and time frame for dispersing MNPs with high-power sonicator was 80%, 22 watts and 20 minutes with 5 seconds on and 5 seconds off, respectively, under the protection of ice to prevent from heat-up. PGS-MNPs suspension was then dropped into the PFA mold to cover the salt template homogeneously. After air dried for 1 hour, the PFA mold was placed into the vacuum oven and kept at 10 torr absolute pressure and room temperature for 1 h to remove all the ethanol residues. The temperature of the vacuum oven was then increased to 120 $^{\circ}\text{C}$ to cure PGS nanocomposite for 48 hours. PGS nanocomposite together with the salt template was taken out from the PFA mold and NaCl was dissolved in deionized water

(DI water) with water change twice in 24 hours. PGS nanocomposite was then air-dried in room temperature for 12 hours.

Characterizations of 3D porous PGS Nanocomposites

Porous PGS nanocomposites were characterized with Canon SLR camera and scanning electron microscope (SEM, Nova NanoSEM 450, FEI, Hillsboro, OR) with spot size 3 and acceleration voltage of 2 kV. The magnification of 100 $\times$ was used to detect the surface features and cross section thickness of porous PGS nanocomposites. Scaffold porosity was quantified with ImageJ based on SEM images of the nanocomposite surface. Alternatively, nanocomposite porosity was also examined with buoyancy balance (Model MS105DU, Mettler-Toledo International Inc. Columbus, OH) by testing scaffold density and calculating with equation (1). X-ray diffraction (XRD) was used to determine the presence of MNPs in PGS.

$$P = 1 - \frac{\rho_s}{\rho} \quad (1)$$

P in the equation stands for porosity of the scaffold, ρ_s is the density of the porous scaffold tested with buoyancy balance and ρ is the density of the original material.

Mechanical testing

The test samples were prepared as rectangular strips of size 12.5 $\times$ 3 $\times$ t mm (t = thickness measured with SEM) for tension test. The tensile properties of the porous

magnetic PGS nanocomposites were characterized using Instron 5969 dual column testing system equipped with 10 N load cell (Instron, Norwood, MA). A 0.005 N preload was applied on the sample prior to tension test and cross-head speed of 10 mm/minute was used until the breakage of the specimen. Three samples were tested of pure PGS and each group of nanocomposites ($n = 3$). The tensile stress-strain curves were plotted from the calculation of the load-extension data. The stress was the load divided by the cross-section area of specimens and the strain was the extension divided by the gauge length of the specimens. The elastic Young's modulus was determined from the linear region of stress-strain curve by fitting a straight line between 0% and 20% strain. Percent elongation until breakage of samples were calculated.

5.2.3 Cell Study of 3D Porous Nanocomposites

Pre-treatment of 3D Porous PGS Nanocomposites

In our previous study, we were able to prove that the presence of PGS residuals caused cell death and morphology change of BMSCs, so a pre-treatment of pure PGS and PGS nanocomposites was critical for cell study. Hence, pre-treatment of porous magnetic PGS nanocomposite was conducted to remove the reaction residuals before cell study. Before the pre-treatment, each type of nanocomposite was cut into 7×7 mm scaffolds and three of them were randomly selected for cell culture. Optical images of all the samples were taken before pre-treatment. The process of pre-treatment was modified from Wang et al. [146]. Briefly, all the scaffolds were soaked serially in 70, 50 and 25% ethanol for 1 hour

each in a laminar flow hood, followed by soaking twice in phosphate buffered saline (PBS) for 1 hour each, and conditioned in DMEM media supplemented with 10% FBS and 1% Penicillin/Streptomycin in standard cell culture environment (i.e., 37 °C, 5% CO₂/95% air, humidified environment) for 24 hours before cell study. Media pH, scaffold dry mass and surface morphology were characterized after the pre-treatment process.

Cell Study of 3D Porous Nanocomposites

Confluent flask of bone marrow-derived mesenchymal stem cells (BMSCs) was washed with 10 mL PBS and then detached by adding 3 mL trypsin (#25200-056, Life Technologies, Carlsbad, CA) and incubating under standard cell culture conditions for 3 minutes. 9 mL media was added subsequently to the flask to neutralize trypsin, and cell suspension in the flask was collected into a 15 mL-centrifuge tube and centrifuged for 5 minutes at 1,000 rpm. The supernatant was aspirated and 5 mL fresh media was added into the tube to resuspend cells. 10 μ L cell suspension was placed onto a small petri dish and 10 μ L of trypan blue solution (#T8154, Sigma-Aldrich, St. Louis, MO) was mixed with the cell suspension. The mixture was loaded into the hemocytometer (#1475, Reichert Bright-Line, Hausser Scientific, Horsham, PA) and cells were counted manually under microscope. Cell suspension volume needed for seeding (10,000 cells/cm²) was calculated and diluted with DMEM as needed. Raschig rings with inner diameter of 6 mm and wall thickness of 1 mm (#50-975-903, Thermo Fisher Scientific, Waltham, MA) were placed on top of each sample prior to cell seeding to prevent the sample from floating. 1 mL of cell suspension

(at a seeding density of 10,000 cells/cm²) was dropped slowly into each of the ring to allow the cell suspension penetrates through the porous scaffolds. The cell study also included one group with magnetic nanoparticles, one group with Raschig rings, one group with cells only, and one group with media only. The amount of magnetic nanoparticles in each of the triplicate wells was 1.03 mg, which was calculated based on the amount of magnetic nanoparticles incorporated into the 7×7 mm PGS+10%MNPs scaffolds. The cell culture well plates were gently placed back into the incubator and cultured for 24 hours and 72 hours under the standard cell culture conditions, and marked as group 2nd and group 4th, respectively.

To compare with the degradation properties of the scaffolds cultured without BMSCs, group 2nd and group 4th was replicated but cultured without BMSCs for 24 hours and 72 hours, and marked as group 3rd and group 5th, respectively.

Cell Culture Media Characterization

After 24 hours and 72 hours of culture, media from each group was collected and media pH was tested with a pH meter (Model H160, Hach, Loveland, CO). Media absorbance was tested with Tecan (Model Infinite M200 Pro, Tecan, Switzerland) to investigate the substances presented in the media.

Cell Morphology and Density Characterization

BMSCs were fixed and stained after 24 and 72 hours of culture. Adherent cells on the 24-well plate and on the scaffolds were both fixed with 4% paraformaldehyde (PFA;

#15714-S, Electron Microscopy Science, Hatfield, PA) solution, stained with Alexa Fluor 488 (#A12379, Life Technologies, Carlsbad, CA) for F-actin and Hoechst 33342 (#H3570, Life Technologies, Carlsbad, CA) for cell nuclei. Then the scaffolds were flipped over and transferred to new 24-well plates and stained with 0.3% Sudan Black B (#152088, MP Biomedicals, LLC, Santa Ana, CA) to prevent light scattering inside the scaffold. After fixing and staining, cells were imaged using a fluorescence microscope (Nikon Ti-S eclipse, Nikon, Japan) and confocal laser scanning microscope (Model Inverted SP5, Leica Microsystems Inc., Buffalo Grove, IL). The images were merged using ImageJ and cell density was calculated. Briefly, for calculating cell density on the plate, five spots in each well were randomly selected for imaging and cell numbers in each image were manually counted. Average cell density in each well was calculated. For calculating cell density on the scaffold, five spots on each scaffold were randomly selected, a z-stacked image was taken for each spot and calculated in the same way.

Scaffold Dry Mass Test of 3D Porous Nanocomposites

Scaffolds were tapped dry with Kimwipes (Kimberly-Clark Worldwide, Inc.) and sample dry masses in group 1st, 2nd, 3rd, 4th and 5th were all measured with Mettler Toledo balance (Model MS105DU, Mettler-Toledo International Inc. Columbus, OH). Test results were recorded and analyzed.

SEM Characterization of 3D Porous Scaffold Surface Morphology

Sample surface morphology in each group was characterized using SEM with spot size 3 and acceleration voltage of 2 kV. The magnification of 350 $\times$ was used to detect the surface features of porous PGS scaffolds post-culture.

Response to External Magnetic Field

The magnetic response of porous magnetic PGS nanocomposite scaffolds before and after cell study were tested with a neodymium magnet embedded in the testing machine named 3D magnet controller which we built in our lab. The scaffolds were able to free-floating on DI water filled in a petri dish with diameter of 8 cm. The bottom surface of the magnet was placed 2.5 cm higher than the top surface of the porous magnetic PGS nanocomposite scaffold and moved in a speed that can direct the scaffold move from one side of the dish to the other side without delay. In another study, the scaffold was moved in a circular motion with the circle diameter of 5 cm in the petri dish. The responding time of scaffold movement was recorded for all groups. The average moving speed of each group of scaffolds was calculated and compared.

Cell Re-plate

Media absorbance test result showed the presence of substances in the media collected after culture, so in a separate repeat study, cell culture media was also collected after 24 hours and 72 hours for cell re-plate. Basically, media from each group was collected

separately, centrifuged at 1,000 rpm for 5 minutes, then the collected substances were re-suspended in 1 mL of fresh media, re-plated to new 24-well plates and cultured at standard conditions for 72 hours.

Statistical Analysis

Experiments were run in triplicate and the data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey highest significant difference post hoc test. Statistical significant difference was considered at $p < 0.05$.

5.3 Results

5.3.1 Characterization of Magnetic Nanoparticles

The synthesized magnetic nanoparticles exhibited spherical morphology, as shown in the scanning electron micrograph (SEM) at a magnification of $50,000\times$ (Figure 5.1A). Quantitative analysis of the SEM image of nanoparticles showed that the average diameter of the magnetic nanoparticles was about 10 nm, which was consistent with the examination of TEM (Figure 5.1B). The lattice spacing was measured to be 0.487 nm, which is indexed to the (111) plane of Fe_3O_4 crystal structure [73]. XRD diffraction pattern confirmed that the synthesized nanoparticles were iron oxide (Figure 5.1C). The major XRD peaks for Fe_3O_4 and $\gamma\text{Fe}_2\text{O}_3$ are very close to one another according to the standard XRD pattern (PDF #19629 and #39-1346, respectively). As shown in Figure 5.1C, major peaks for Fe_3O_4 were present, and the minor peaks for $\gamma\text{Fe}_2\text{O}_3$ were absent, indicating the synthesized nanoparticles were most likely to be Fe_3O_4 phase. Hysteresis loop of the magnetic nanoparticles

showed a low coercivity and low magnetization after the removal of external magnetic field (Figure 5.1D), which is in the range that is usually considered to be superparamagnetic [152].

5.3.2 Characterization of PGS Nanocomposites

Optical, SEM and XRD characterization

Optical images of PGS composite scaffolds with different ratio of MNPs exhibited a color change. With MNPs ratio from 0% to 10%, the color of PGS scaffolds range from transparent to light brown till completely black (inset in Figure 5.2A'-E'). SEM analysis showed the presence of interconnected pores with main pore size corresponding to the NaCl salt size which ranged from 125-150 μm . The pore distribution was consistent on the surface (Figure 5.2A-E) and cross section for all groups (Figure 5.2A'-E').

The porosity of each group of scaffold was tested with buoyancy balance and analyzed with ImageJ. Analysis results were averaged by three samples in each group. The buoyancy balance test results were 39.7%, 52.7%, 60.1%, 44.7% and 49.3% for PGS+0%, 0.5%, 1%, 5% and 10% scaffolds, respectively. And the ImageJ analysis results were 43.7%, 53.3%, 58.1%, 35.5% and 36.4% for PGS+0%, 0.5%, 1%, 5% and 10% scaffolds, respectively. There was no significant difference in the porosity tested between each group and no significant difference in the porosity analyzed with two methods.

The cross section of each group of scaffold was imaged with SEM and the thickness of each group of scaffold was analyzed with ImageJ (Figure 5.2A'-E'). Analysis results were

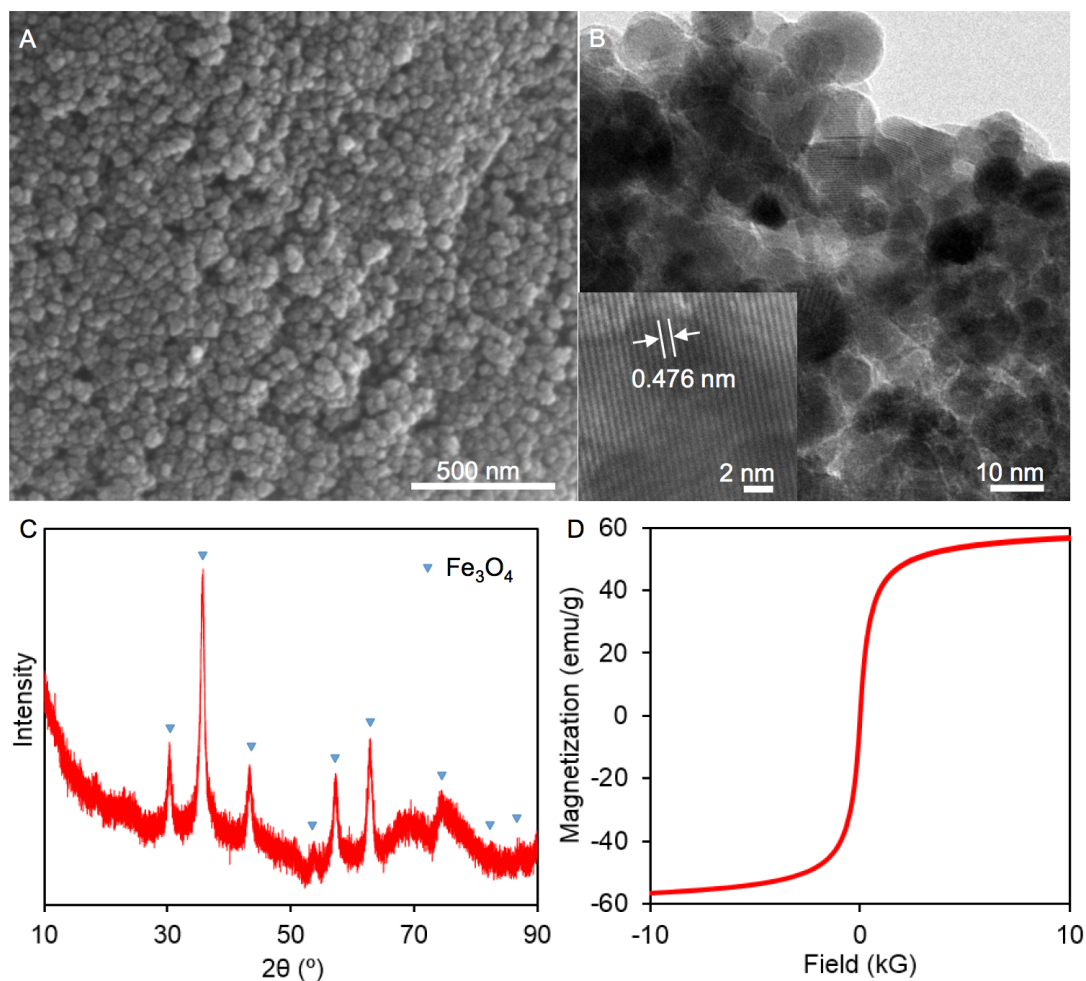


Figure 5.1: Characterization of the MNPs used in the magnetic nanocomposites. (A) SEM image of the MNPs (30% PVA-coated Fe_3O_4 nanoparticles). Original magnification = 50,000 $\times$, scale bar = 500 nm. (B) TEM image of the MNPs. Original magnification = 150,000 $\times$, scale bar = 10 nm. Inset: high-magnification TEM image showing the lattice planes. The interplanar distance was measured to be 0.476 nm, which is indexed to be the (111) plane of Fe_3O_4 crystal structure. Original magnification = 740,000 $\times$, scale bar = 2 nm. (C) X-ray diffraction pattern of the MNPs, step size = 0.0066°. Fe_3O_4 standard pattern JCPDS #65-3107. (D) Hysteresis loop of the MNPs, showing the property of superparamagnetism.

averaged by five samples in each group (Table 3). The thickness of each group of samples were 324.4 μm , 292.5 μm , 344.2 μm , 212 μm and 268.8 μm for PGS+0%, 0.5%, 1%, 5% and 10% scaffolds, respectively. There was no significant difference between the thickness of each group of scaffold.

XRD spectrum of pure PGS only showed a broad peak which indicated the absence of crystal structures, while for mPGS, it was easy to tell the presence of MNPs with the narrow and sharp peaks in different angles. And with the increasing ratio of MNPs in magnetic PGS composites, the peak intensities for MNPs also increased (Figure 5.2F).

Mechanical characterization

The stress-strain curves of 3D porous magnetic PGS nanocomposites were presented in Figure 5.3A and Youngs modulus and percent elongation of the nanocomposites were calculated from the stress-strain curves and illustrated in Figure 5.3B. From test results, the tensile Youngs modulus of pure PGS was 0.055 ± 0.002 MPa and PGS can be elongated to about two times of its original length without rupture. The values presented here are mean $\pm$ standard error of mean. With the addition of 0.5% MNPs, the Youngs modulus of PGS nanocomposite increased to 0.231 ± 0.016 MPa. However, the Youngs modulus did not keep increasing with higher amount MNPs, only higher than the Youngs modulus of pure PGS. The trend of percent elongation change was the opposite. The addition of MNPs to pure PGS resulted in increase in tensile modulus and decrease in elongation compared to pure PGS network. The Youngs modulus of 0.5% group was significantly higher than

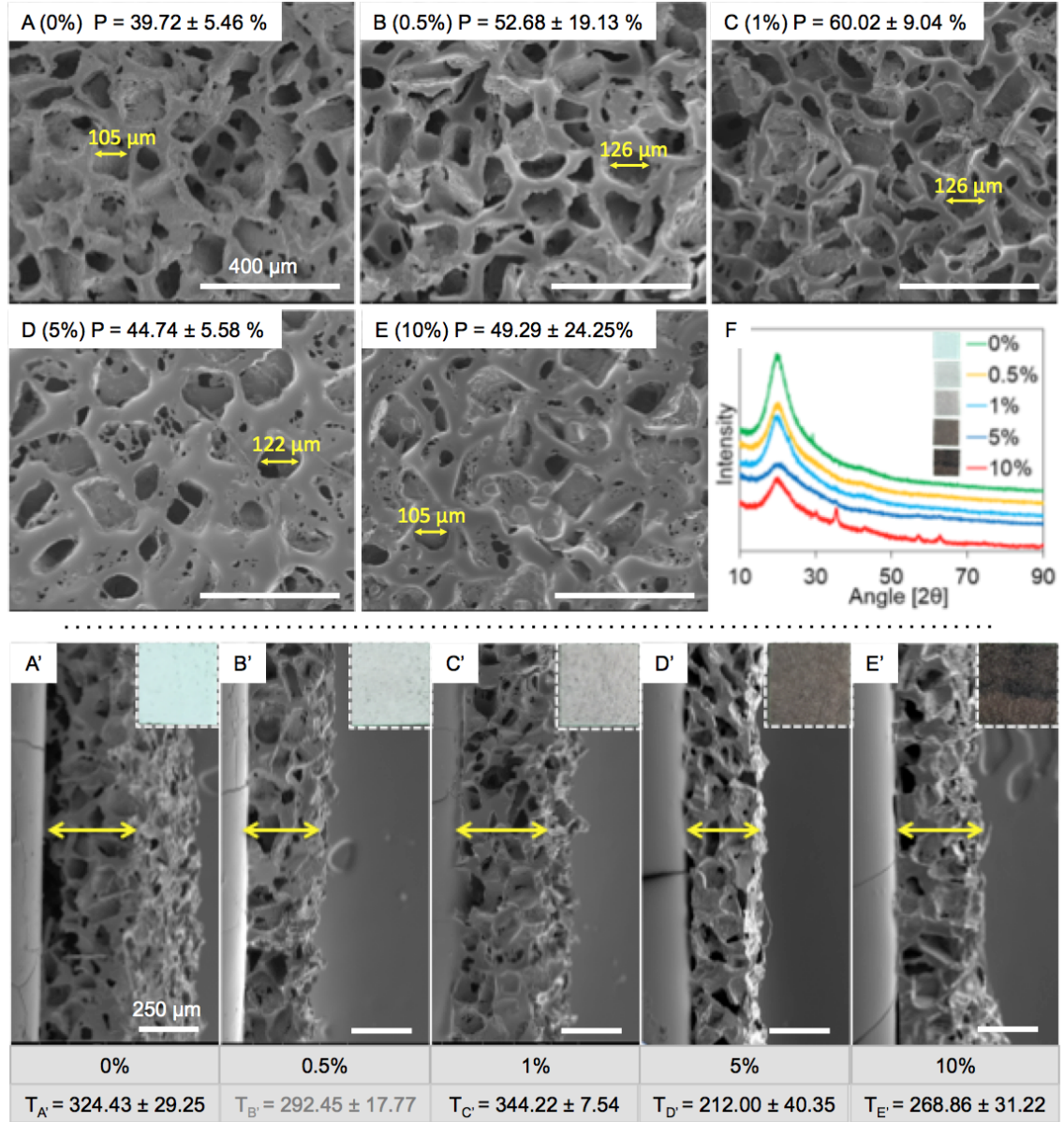


Figure 5.2: Characterization of magnetic nanocomposites with increasing weight percent of MNPs. Each group of magnetic nanocomposite was referred to by the weight percent of MNPs incorporated in it. SEM images showing the surface and cross section of (A, A) PGS+0%MNPs (0%), (B, B) PGS+0.5%MNPs (0.5%), (C, C) PGS +1%MNPs (1%), (D, D) PGS+5%MNPs (5%) and (E, E) PGS +10%MNPs (10%). (A-E) Original magnification =100 $\times$, scale bar = 400 μ m. (A'- E') Original magnification = 50 $\times$, scale bar = 250 μ m. Yellow arrow indicates the cross section of each group of scaffold. (F) X-ray diffraction pattern of the magnetic nanocomposites. Inset in (F): photographs of corresponding magnetic nanocomposites. Scale bar = 5 mm.

the rest of the groups. The Young's modulus and percent elongation of 3D porous pure PGS and all magnetic PGS nanocomposites were consistently lower than that of the 2D pure PGS and non-porous magnetic PGS nanocomposites that was reported by our group previously, which was expected due to the porous structure.

5.3.3 Cell Study of 3D Porous Nanocomposites

Cell Morphology on the Nanocomposites

Because of the surface morphology of the scaffolds, cells grow on the scaffold surface were prefer to settle on the pore area and were hard to be imaged in one focal plane, so the z-stack technique was used to obtain a full image of all cells grow in one spot (Figure 5.4A). Cell morphology were similar to the cells grow on the tissue-culture plate but were smaller and elongated.

Cell Morphology on the Plate

Those cells grow on the plate were in a normal morphology, which were irregular star-shaped and with multiple pseudopodia, and even those cells grow near to the edge of the scaffold were still in a normal morphology and were able to be imaged in one focal plane (Figure 5.4B).

Cell Density on the Nanocomposites

Cell densities of cells grow on the scaffold after 24 hours and 72 hours culture are shown in Figure 5.5A. There was an increasing trend in the cell density from PGS+0%,

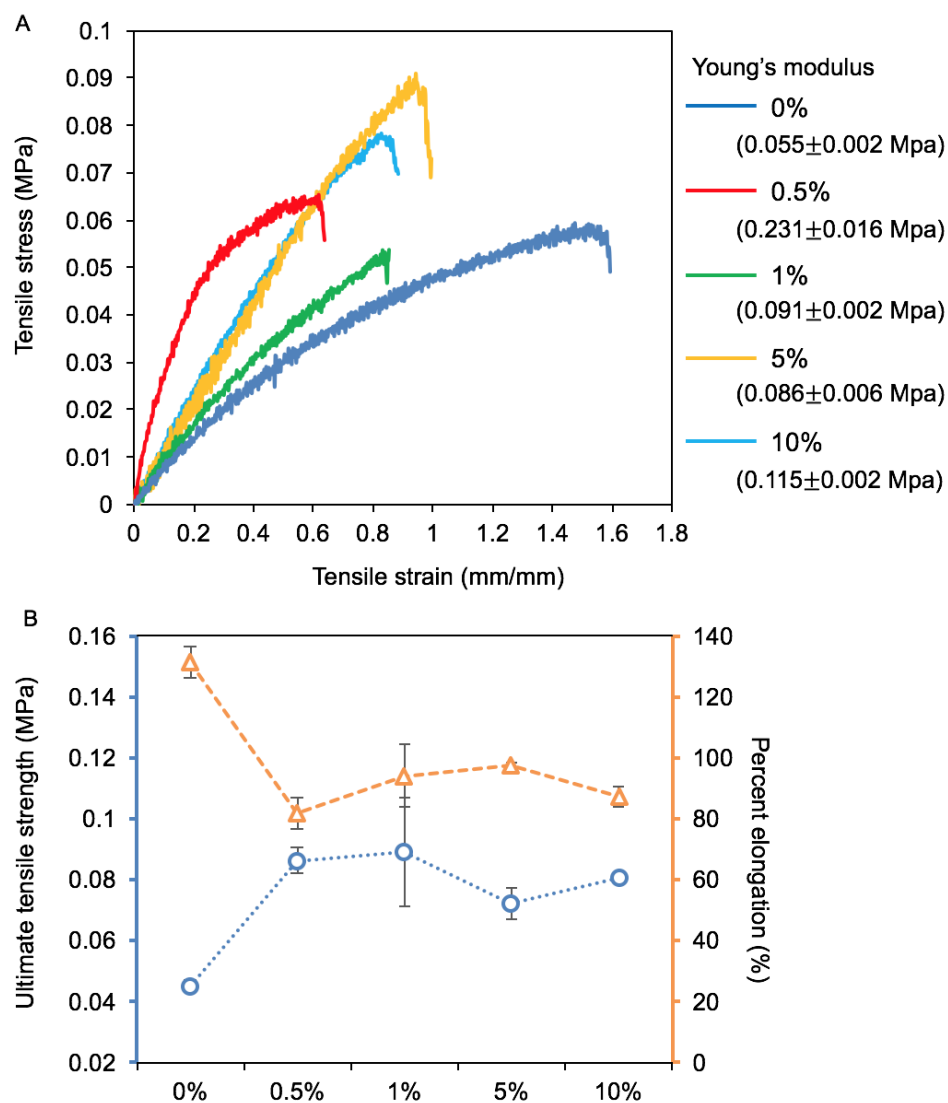


Figure 5.3: Tension test of magnetic PGS nanocomposites. (A) Tensile stress-strain curves for 0, 0.5, 1, 5 and 10% MNPs-incorporated 3D porous PGS nanocomposites with Young's modulus labeled on the right. (B) Ultimate tensile strength and percent elongation of 0, 0.5, 1, 5 and 10% MNPs-incorporated 3D porous PGS nanocomposites. Values are means $\pm$ standard error of mean, $n = 3$.

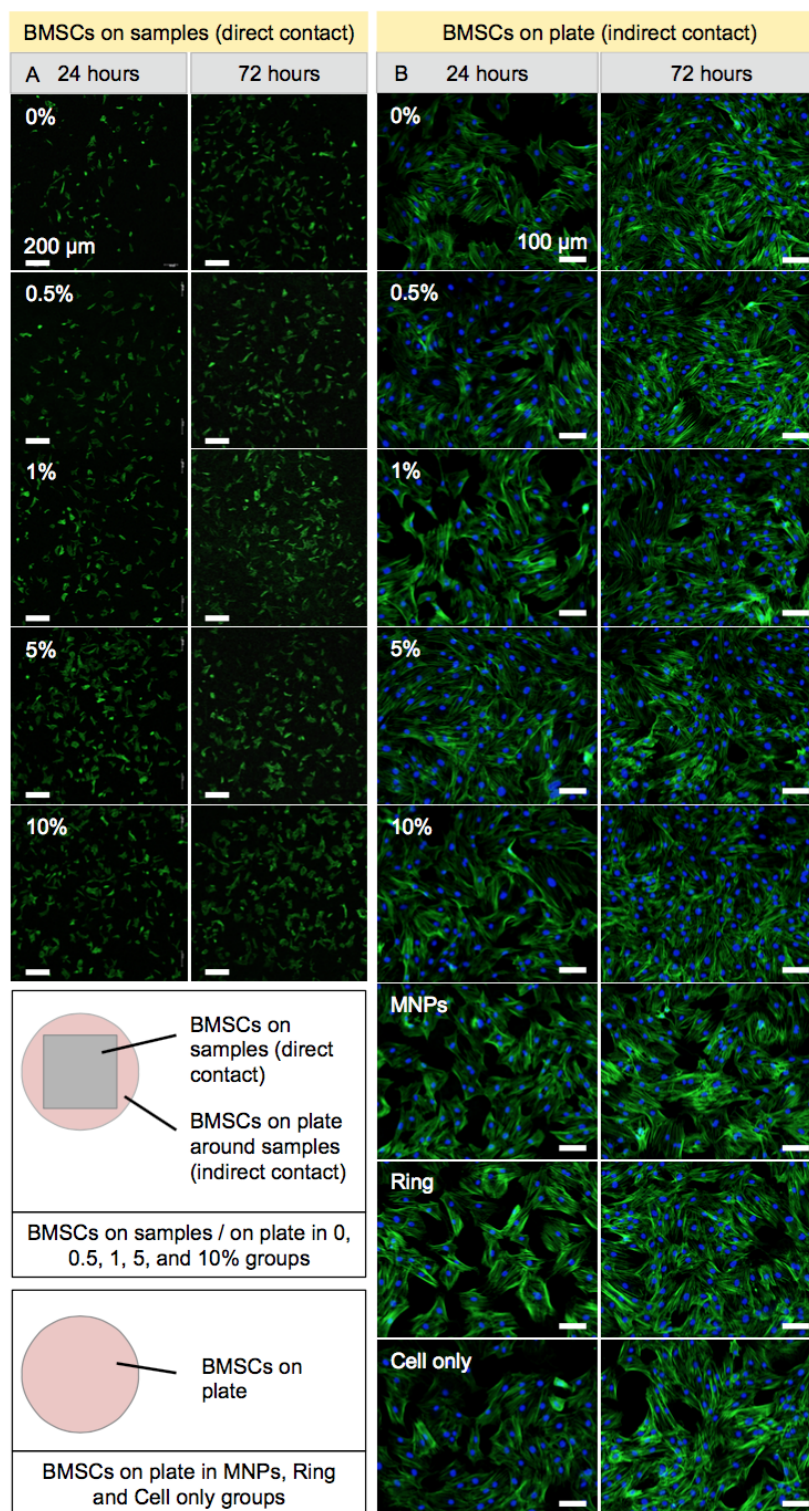


Figure 5.4: The morphology of BMSC after 24 and 72 hours of culture with magnetic nanocomposites. (A) BMSC morphology on the nanocomposites (direct contact) after 24 and 72 hours. (B) BMSC morphology on the plate around the nanocomposites (indirect contact) after 24 and 72 hours.

0.5%, 1%, 5% to PGS+10% scaffold. However, after 72 hours of culture, cell densities in each group were more similar. Cell density in the 0% and 0.5% group after 24 hours of culture were lower than the seeding density (10,000 cells/cm²) while the rest of the three groups were higher than seeding density. After 72 hours of culture, cell density in all groups was higher than seeding density but the increase from 24 hours was not significant within each group.

Cell Density on the Plate

Cell densities on the plate were all slightly higher than the seeding density after 24 hours of culture and almost doubled after 72 hours of culture. Cell densities were similar in most groups after 24 hours and 72 hours of culture except the ring group, which had a significant lower cell density than any other groups after 72 hours of culture (Figure 5.5B).

Overall Cell Density in Each Well

The overall cell density in each well was quantified based on the cell density on the scaffold and cell density on the plate around the scaffold in one well. Results showed that the overall cell density trend was similar to the trend of cell density just on the plate and only the ring group had a cell density significantly lower than the cell density in all other groups after 72 hours of culture (Figure 5.5C).

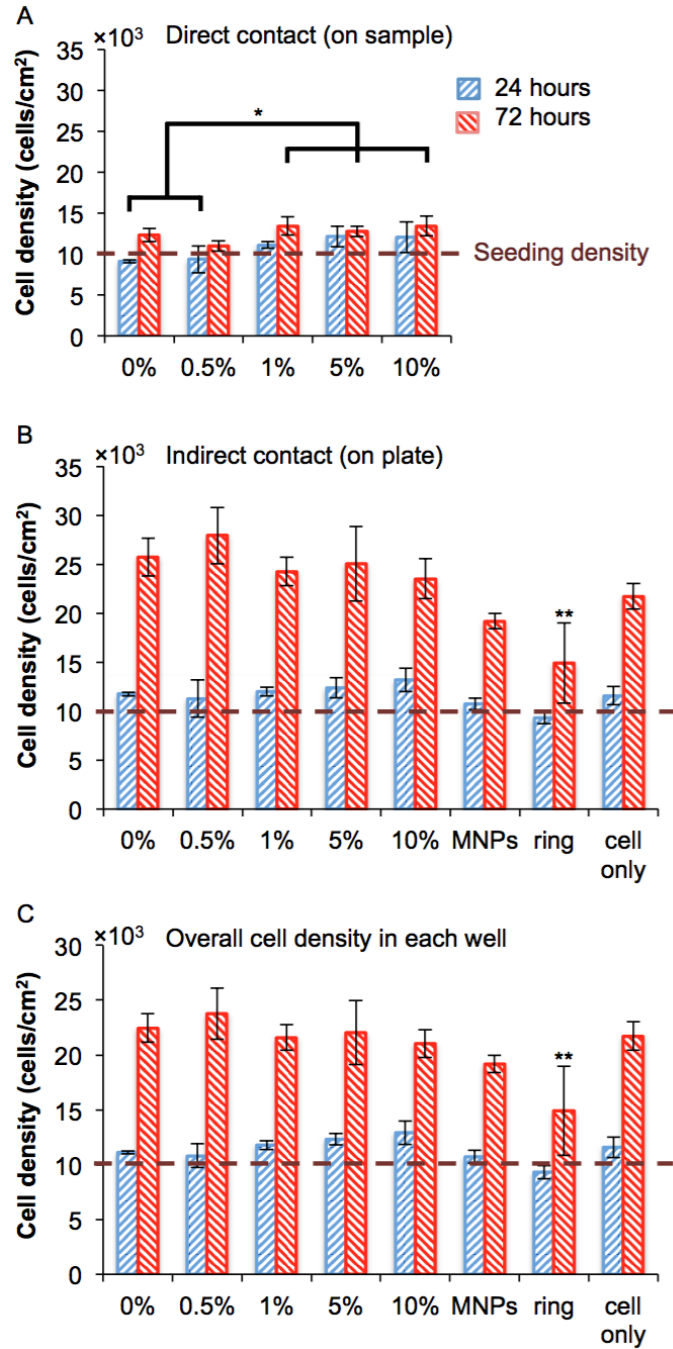


Figure 5.5: The density of BMSC after 24 and 72 hours of culture with magnetic nanocomposites. (A) BMSC density on the nanocomposites after 24 hours and 72 hours. (B) BMSC density on the plate around the nanocomposites after 24 and 72 hours. (C) Overall BMSC density in each well after 24 and 72 hours. * $p < 0.05$, ** $p < 0.01$.

Change of Media pH

The media pH of each group was similar and in the range of 8-8.6. There was a slight increase of pH in the group of cells cultured with scaffold for 72 hours when compare to the group of cells cultured with scaffolds for 24 hours, however, no significant pH change was detected in all groups (Figure 5.6A).

Sample Dry Mass Loss

Sample dry mass loss increased from 0% group to 0.5% group and then decreased from 0.5% group to 10% group in the same time point. Dry mass loss of the same type of sample was similar in different time points cultured with or without BMSCs except the 0.5% group, which had the highest dry mass loss when cultured without BMSCs for 24hours (Figure 5.6B).

SEM Characterization of the Scaffold Surface after culture

SEM images showed the presence of degradation evidences on the PGS nanocomposite scaffolds. The top surface of the scaffolds still possesses smooth morphology while the wall of the pores showed the most existence of square and stair-shaped features, which were resulted from the surface degradation of PGS nanocomposites. Those features presented in all the groups and similar in different time points regardless of cultured with or without BMSCs (Figure 5.7).

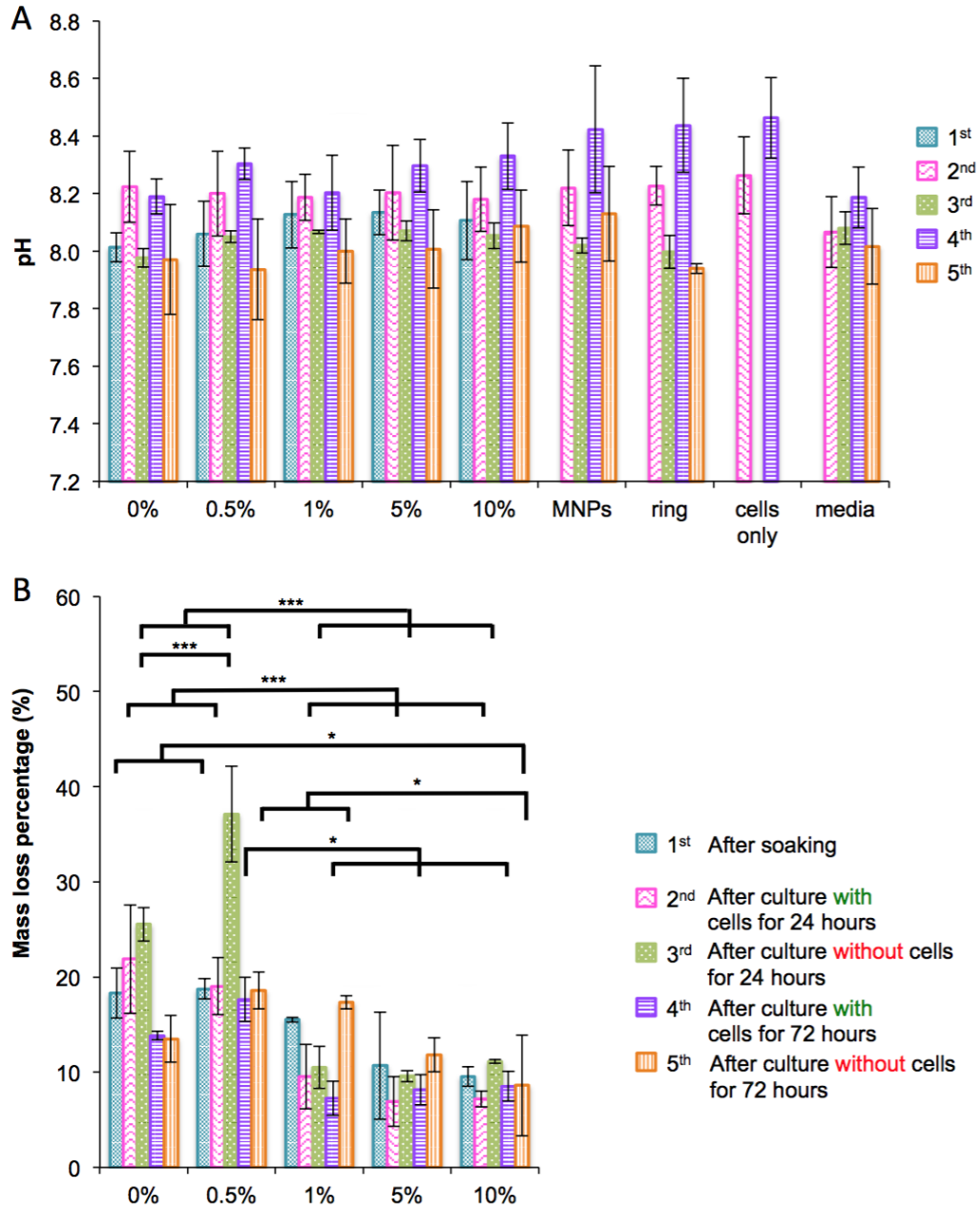


Figure 5.6: Media pH and mass loss percentage of each group after culture for different time period. (A) Media pH of each study group after culture. (B) Nanocomposite mass loss percentage of each group after culture. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

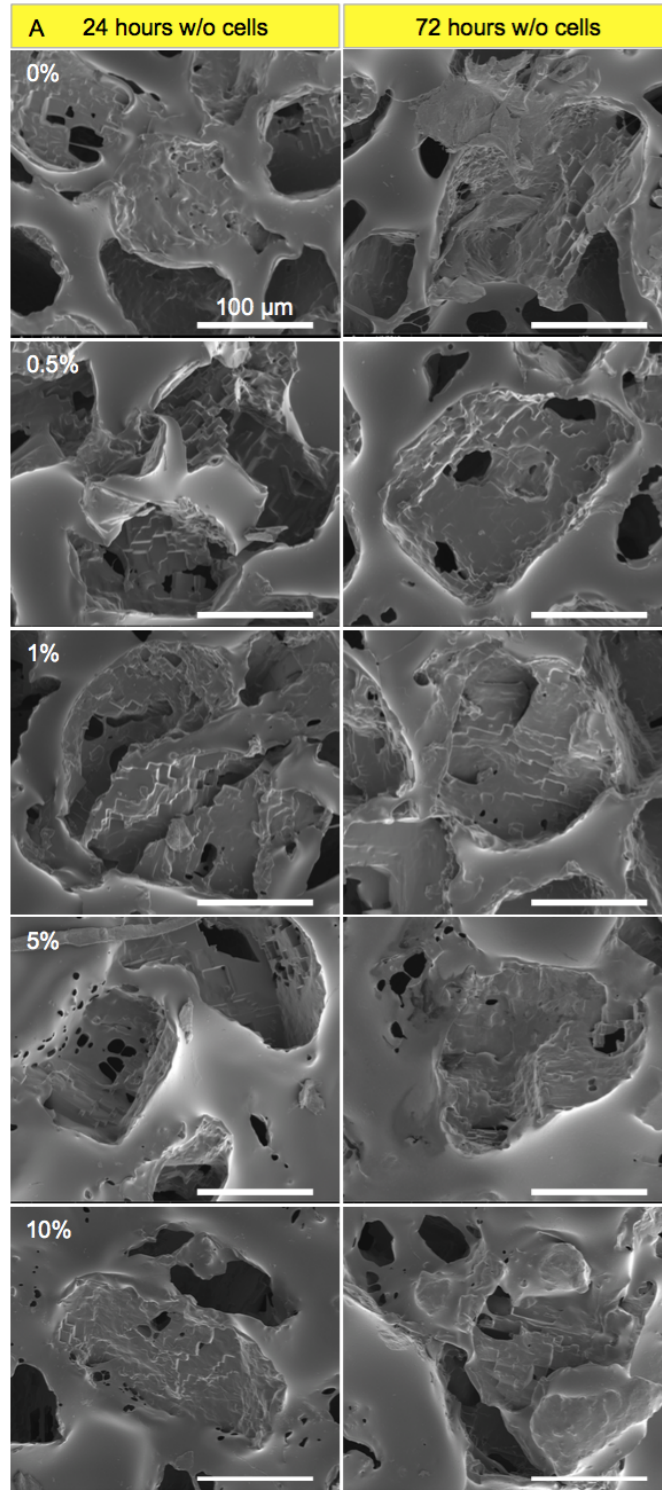


Figure 5.7: (A) Surface morphology of nanocomposites after cultured in media without cells for 24 hours and 72 hours. Original magnification = $350\times$, scale bar = $100\text{ }\mu\text{m}$.

Response to External Magnetic Field

Pure PGS scaffold could not be attracted by an external magnet due to the absence of magnetic materials incorporated, while mPGS scaffold with MNPs ratio of 0.5%, 1%, 5% and 10% showed response to magnet after culture with or without cells for 24 hours and 72 hours. And with the increase of MNPs ratio, the response speed of mPGS scaffold to the magnet was also increased, from 0.5 to 1, 3.5 and 6 cm/s. The response speed of 5% and 10% group were significantly higher than 0.5% and 1% group in each time point and the response speed of 10% group was significantly higher than it in the 5% group in each time point. It was able to see that with the increasing ratio of MNPs, the acceleration of mPGS composite scaffold towards the magnet was also increased (Figure 5.8).

Cell density after re-plate

Cell density in 0% group was higher in the 24 hour-time point when compared to it in the 72 hour-time point. Cell density was gradually increased within the 0.5%, 1%, 5% and 10% group from 24 hour-time point to 72 hour-time point, and all the densities were either similar or higher in the sample groups when compare to the MNPs group and cell only group. The cell density in the MNPs group were higher in the 72 hour-time point when compare to the 24 hour-time point while the cell only group was the opposite. Surprisingly, the ring group had a significantly higher cell density in both 24 hour- and 72 hour-time point, which reason is not clear yet (Figure 5.9). However, the cell density after re-plate

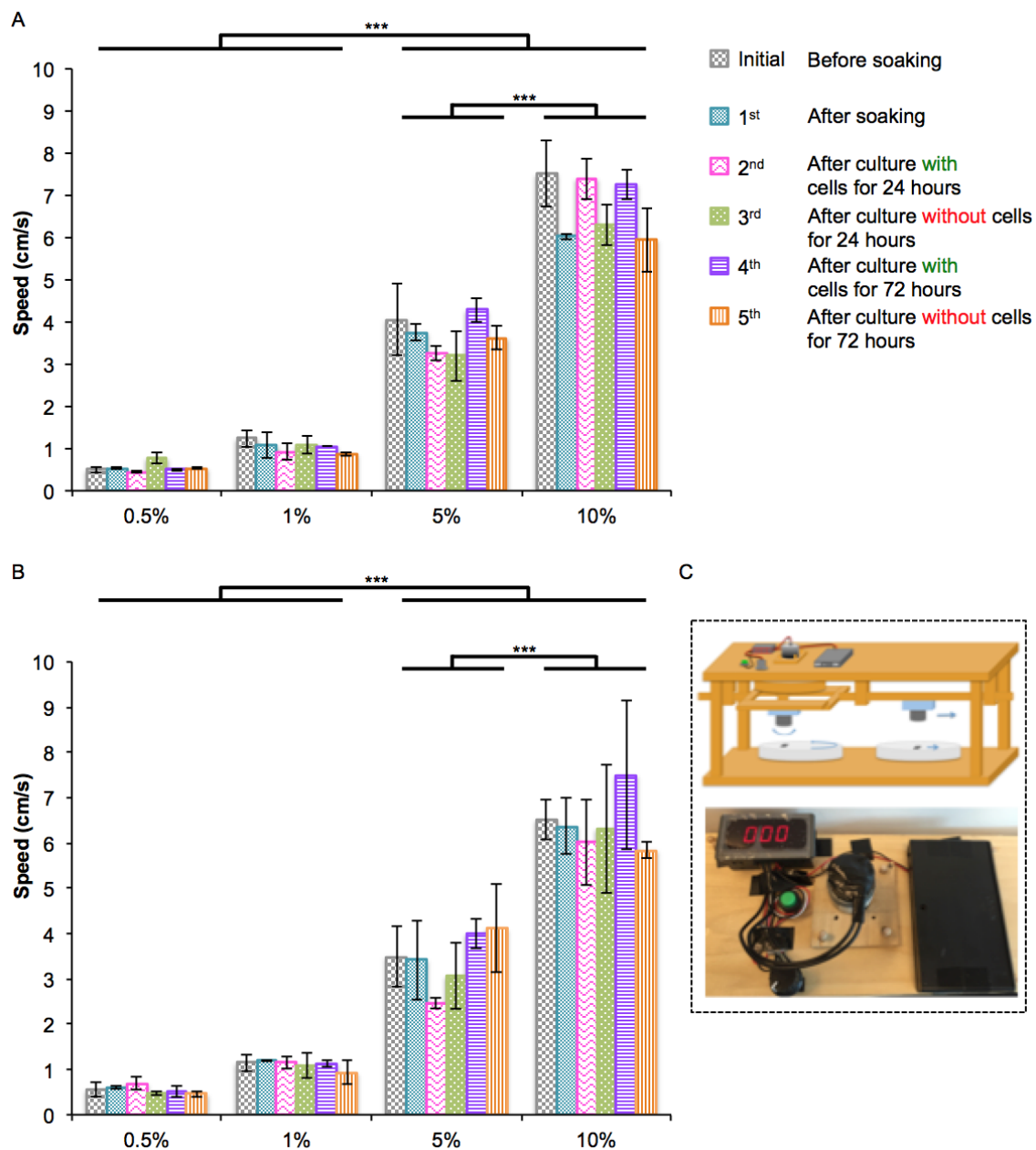


Figure 5.8: Remote guidance of the magnetic nanocomposites using a 3D magnetic controller. (A) Circular motion and (B) linear motion of each group of magnetic nanocomposite under the guidance of 3D magnetic controller. (C) The schematic design of the 3D magnetic controller (upper) and the photograph of the control unit (lower). *** $p < 0.001$.

showed evidence for the degradation of scaffolds and only those cells attached to the surface of the scaffolds were carried out by the degraded scaffolds.

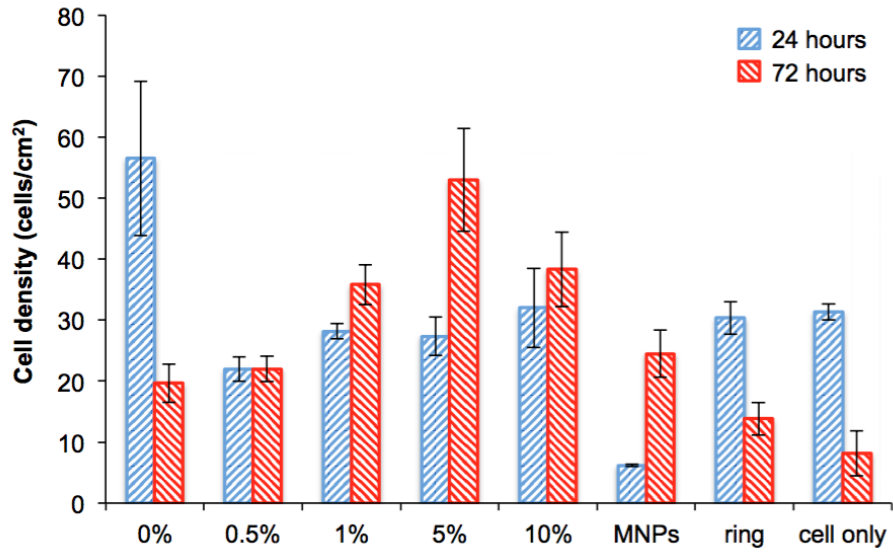


Figure 5.9: BMSC density after 24 hours and 72 hours of re-plate culture.

5.4 Discussion

5.4.1 Magnetic Nanoparticle Characterization

The size of the magnetic nanoparticles synthesized in lab was around 10 nm, which is in the range of the size reported can be ultimately excreted renally and cause less harm to human body [169]. The clear lattice plane showed the existence of crystal structures supported by the XRD spectrum. The overlapped hysteresis loop showed the low coercivity and superparamagnetic properties of the magnetic nanoparticles, which is

optimal to be cleared out from human body afterwards and easy to be intake and go through the metabolism of BMSCs.

5.4.2 Characterization of 3D Porous PGS Nanocomposites

All five groups of scaffolds showed porosity close to the designated value and the surface roughness was greatly increased after salt-leaching method. The pore size was around 125-150 μm , which was the same as the size of the salt. BMSCs are usually in the size of around 100 μm , so the designed pore size would allow the cells to grow inside the pores and penetrate to the center of the scaffold through the inter-connected pores. The z-stacked image from fluorescence microscope confirmed this. The thickness of the scaffolds was in the range of 212 to 324 μm , and the fluorescence images showed the cells are able to penetrate into and grew inside the scaffolds. XRD spectrum confirmed the presence of gradually increased MNPs ratio from 0% to 10% scaffold.

5.4.3 3D Porous magnetic PGS nanocomposites characterizations

According to the results of tension tests, the 3D porous magnetic PGS nanocomposites demonstrated higher Young's modulus compared to pure PGS and 0.5% group was significantly higher than pure PGS group. The stress-strain curves exhibit nonlinear stress-strain behavior, which is similar to that of non-porous PGS and typical for soft elastic materials [148]. The porous-structured PGS nanocomposites preserved elastic property of PGS, which was due to the covalent crosslinking and hydrogen bonding interactions of hydroxyl groups attached to the backbone of PGS [148]. It is speculated that, the MNPs could

crosslink with PGS polymer through the covalent crosslinking of hydroxyl groups reside on the surface of MNPs with the hydroxyl groups on the backbone of the polymer, which may contribute to the increasing of mechanical properties of the magnetic PGS nanocomposite.

5.4.4 Cell Study of 3D Porous Magnetic PGS Nanocomposites

Morphology of the cells attached on different groups of PGS nanocomposite was similar and close to the normal cell morphology, which was irregular star-shaped and with multiple pseudopodia. The cell density on the scaffold was also similar. Morphology of the cells on the plate around the scaffolds was similar to those cells attached to the plate in the control group. Cell density on different groups of scaffolds was similar in 24 hour-time point with an increasing trend from PGS+0%MNPs scaffold to PGS+10%MNPs scaffold, but the trend disappeared in 72 hour-time point. Changes in cell densities indicated that the 10% group was favored by cells for initial attachment, but cells proliferate in a similar speed afterwards. The lower increase of cell densities on the scaffold when compare to the density increase on the plate was resulted by the surface degradation of the scaffolds.

The cell density in the ring group at the 24 and 72 hour-time point was significantly lower than the cell density in all other groups at the same time point. While after re-plate, the cell density in the ring group was significantly higher than all other groups, which means some cells were still alive even without attachment in the ring group and survived in the re-plate process.

The introduce of porous structure impaired the mechanical properties of magnetic PGS nanocomposites but increased cell adhesion density and promoted cell proliferation on the surface and inside the nanocomposites. The Youngs moduli of the magnetic PGS nanocomposites are slightly lower than cartilage (0.7-15.3 MPa), nevertheless, the preparation of nanocomposites with increased stiffness requires more crosslinking, which accompanies with smaller pore size or lower porosity, thus could affect the diffusion of oxygen and necessary nutrients for cells [170]. Further research should be done on this to define the ideal parameters for applications in soft tissue repair and utilize its magnetic property that enables remote magnetic manipulation of implanted scaffolds.

5.4.5 Degradation of Porous Magnetic PGS Nanocomposites

The feature of surface degradation was obvious after the scaffolds had been soaked in media for 24 hours and 72 hours. The pore walls showed the presence of layered surface, which was considered the evidence of surface degradation of the PGS nanocomposites. Scaffold dry mass was still considered the best way of showing scaffold mass change because the pores in the scaffold will affect the buoyancy mass test results and give a large error. The overall decreased dry mass loss indicated a slower degradation rate with a higher MNPs incorporation ratio. However, the higher dry mass loss of 0.5% when compare to 0% scaffold, suggested that a small amount incorporation of MNPs would help increase the degradation rate. The media absorbance was consistent with the dry mass loss, suggesting that the 10% group has the lowest degradation rate than all the other groups and the higher the MNPs

incorporation ratio, the slower the degradation rate. The results of the re-plate cell study suggested that the higher absorbance was resulted by the degraded scaffold and/or the dead cells. Live cells degraded out together with the scaffold in each group were similar and most of the cells stayed inside the pores and was not degraded.

The dry mass loss of the 1, 5 and 10% were all similar. It might because there were interactions between the cell culture media and the 0% and 0.5% group helped the deposition of some certain substances. Since there were no significant differences between the 72 hours of culture with and without cells, the factor of cell function will be eliminated.

5.4.6 Magnetic Response of Porous Magnetic PGS Nanocomposites

Test results of magnetic response showed that all magnetic nanocomposite scaffolds maintained their magnetic response ability after culture and the same type of scaffold had consistent response at each time point. Magnetic response ability increased with the increase of MNPs ratio. 1% was not significantly higher than 0.5%, while 5 and 10% were significantly higher than both the 0.5% and 1%. Also 10% was significantly higher than the 5% group.

5.5 Conclusions

Porous structure helped increase BMSC attachment and BMSCs favors the porous area. BMSCs grew on different group of nanocomposites did not show significant differences in their morphology and density and BMSCs were able to migrate inside the scaffold and survive for 72 hours. However, surface degradation of the nanocomposites resulted in the

low increase of BMSC density grew on each group of nanocomposite after 72 hours when compared to the 24 hours of culture. Experiments showed that small amount of MNPs incorporation such as 0.5 wt% would increase the degradation rate of PGS while a ratio higher than that would help decrease the degradation rate. MNPs can be used to adjust the degradation rate of PGS so as to be compatible with the new tissue growth rate of the defect tissue site. The preserved magnetic responsive ability of the nanocomposites after culture made it possible to react to external magnetic field stimuli even after implantation for several days and this would help the study on the synergistic effects of external magnetic fields and MNPs on stem cell proliferation and differentiation.

5.6 Acknowledgements

We would like to thank Mohammed Aldosary and Dr. Jin Shi for their kind help with hysteresis loop test. We thank Central Facility for Advanced Microscopy (CFAMM) at the University of California, Riverside, for the use of SEM, and Ilkeun Lee and Dr. Krassimir Bozhilov for the training on SEM. We thank Enhancing STEM Transfer and Success through the STEM Pathways Program grant (#P031C110131) for the support of undergraduate student researcher (J.S.).

Chapter 6

The Comparison of Two Static Magnetic Fields on Bone Marrow-Derived Mesenchymal Stem Cell Proliferation and Morphology

6.1 Introduction

As introduced in Chapter 1, all living organisms, including animals, plants and microorganisms are continuously exposed in the large scope of magnetic field (MF) generated by our planet. With the development of high technology, there are more and more magnetic fields generated by those high-tech devices. However, the biological effects of those magnetic fields on living organisms are still not very clear [21].

In this study, we will be focusing on the study of two moderate static magnetic fields generated by a permanent magnet and the effects of those fields on bone marrow

derived stem cell (BMSC) proliferation and morphology. BMSC is a kind of multipotent stem cell in bone and they have the ability to complement the native cell population and to help tissue maintenance [153]. They primarily contain mesenchymal stem cells and hematopoietic stem cells and play a significant role on implant and tissue integration [154].

6.2 Materials and Methods

6.2.1 Manufacture of the Magnetic Device

The magnetic device was designed with a neodymium permanent magnet (#DEX0, K&J Magnetics, Inc., Pipersville, PA) in the center and supported by aluminum. The size of the magnetic device ($6 \times 4 \times 1$ inch) is designed to be a bit bigger than the size of standard tissue culture plate ($5 \times 3.37 \times 0.57$ inch) to secure the plate on top of it. The field intensity under each well of the 48-well tissue culture plate was tested with LakeShore 421 gaussmeter (Lake Shore Cryotronics, Inc. Westerville, OH). The schematic is shown in Figure 6.1. When conduct the cell study, the magnetic device will be put underneath the tissue culture plate to provide consistent and static magnetic field (Figure 6.1, 6.2).

6.2.2 Preparation of DMEM

Dulbeccos modified Eagles medium powder (DMEM, #SLBC9050, high glucose, D5648, Sigma-Aldrich, St. Louis, MO) and sodium bicarbonate (NaHCO_3 ; #S5761, Sigma-Aldrich, St. Louis, MO) were weighed out and dissolved in DI water to obtain a media with DMEM and NaHCO_3 concentration of 13.4 and 3.7 mg/mL, respectively. The pH of media

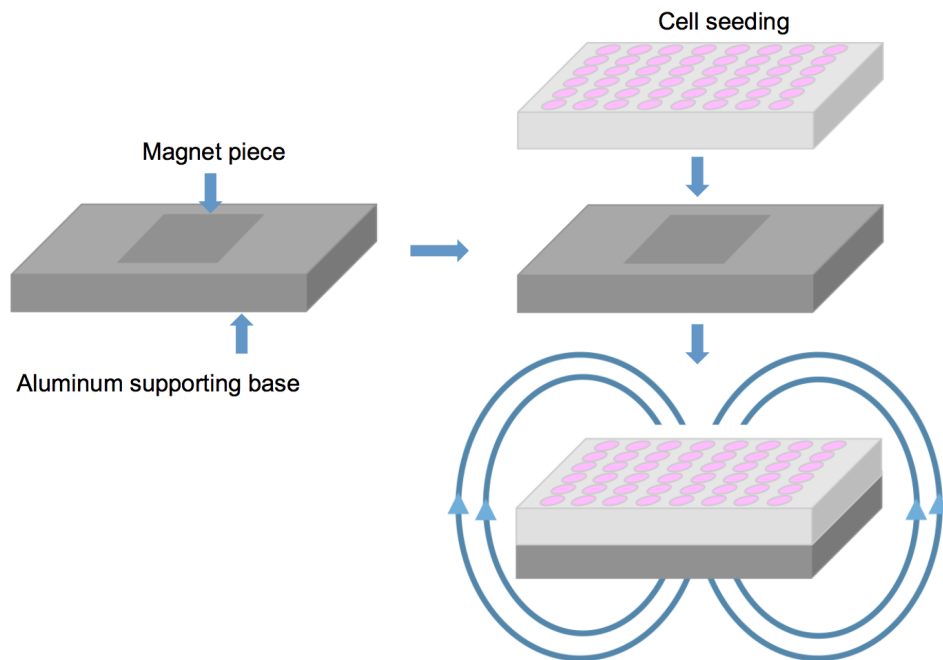


Figure 6.1: The design of magnetic device and how to use it for cell study.

was adjusted to 7.1 by adding 1 M hydrogen chloride (HCl) solution. The media were sterilized using a 0.2 μm filter (#596-0020, Thermo Fisher Scientific, Inc., Waltham, MA). For BMSC culture, the media were supplemented with 10% fetal bovine serum (FBS; HyClone, #SH30910, Thermo Fisher Scientific Inc., Waltham, MA) and 1% penicillin/streptomycin (P/S; HyClone, #SV30010, Thermo Fisher Scientific, Inc., Waltham, MA). Hereafter, DMEM + NaHCO_3 + 10% FBS + 1% P/S is referred to as complete DMEM (cDMEM).

6.2.3 Cell Culture on the Magnetic Device

The magnetic device was sprayed down with 70% ethanol. A magnetic pen was used to check the incubator to make sure there were no other magnetic factors. A confluent flask of bone marrow-derived mesenchymal stem cells (BMSCs) was washed with 10 mL

PBS, detached by adding 3 mL trypsin (#25200-056, Life Technologies, Carlsbad, CA) and incubated under standard cell culture conditions (i.e., 37 °C, 5% CO₂/95% air, humidified, humidified environment) for 3 minutes. 9 mL complete DMEM (cDMEM) was added to the flask after 3 minutes to neutralize trypsin and the cell suspension was collected into a 15 mL-centrifuge tube and centrifuged for 5 minutes at 1,000 rpm. The supernatant was aspirated and 5 mL fresh cDMEM was added into the tube to resuspend the cells. 10 μ L cell suspension was dropped onto a small petri dish and then 10 μ L of trypan blue solution (#T8154, Sigma-Aldrich, St. Louis, MO) was mixed with the cell suspension. The mixture was loaded into the hemocytometer (#1475, Reichert Bright-Line, Hausser Scientific, Horsham, PA) and cells were counted manually under microscope. Cell suspension volume needed for seeding (10,000 cells/cm²) was calculated and diluted with cDMEM as needed. 1 mL of cell suspension (at a seeding density of 10,000 cells/cm²) was pipetted into all study wells of the 48-well plate, and another 6 mL cell suspension was added into six wells of a second 48-well plate for control experiment. The first cell culture plate was placed on top of the magnetic device at the designated spot and both the culture plate and magnetic device was gently placed back on the first layer of the incubator. The second plate was placed on the second layer of the incubator to avoid the influence of the magnetic device. Both plates were under the standard cell culture conditions and the cells were cultured for 72 hours.

6.2.4 Cell Density and Morphology Analysis

After 72 hours, all the cDMEM were removed from each well. Non-adherent cells were removed by washing with PBS. Adherent cells on the 48-well plate were fixed with 4% paraformaldehyde (PFA; #15714-S, Electron Microscopy Science, Hatfield, PA) solution, stained with Alexa Fluor 488 (#A12379, Life Technologies, Carlsbad, CA) for F-actin and 4',6-diamidino-2-phenylindole (DAPI; #D3571, Life Technologies, Carlsbad, CA) for cell nuclei. After fixing and staining, cells were imaged using a fluorescence microscope (Nikon Ti-S eclipse, Nikon, Japan) and the images were merged using ImageJ, cell density in each well was calculated as well. Briefly, five random spots in each well were imaged and the number of cells in each image was quantified. The cell density in each image area (or spot) was calculated using equation 1, and the average cell density in each well was calculated using equation 2.

$$\text{cell density in spot } i = \frac{\text{cellnumberinimage}i}{\text{areaofimage}i} \quad (1)$$

$$\text{average cell density} = \frac{\sum_1^i \text{celldensityinspot}i}{5} \quad (2)$$

in which i represents the random imaging spot from 1 to 5 in each well. Image area is in the unit of cm^2 .

6.2.5 Statistical Analysis

Experiments data were analyzed in R using one-way analysis of variance (ANOVA) followed by the Tukey highest significant difference post hoc test. Statistical significant difference was considered at $p < 0.05$.

6.3 Results

6.3.1 Magnetic Field Strength Test Result of SMF Device

Magnetic field intensity decreases fast with respect to the distance from the surface of the magnetic device. The thickness of the bottom of the tissue culture plate is about 1 mm, so the intensities 1 mm above the surface of the magnetic device were tested because that is the height where the cell will grow on. Results showed that the central part, where located the permanent magnet had an average intensity of static magnetic field of 340 ± 20 mT, and the outside part around the permanent magnet had an average field intensity of 14 ± 16 mT. Those two fields were all in the range of moderate magnetic fields. Testing result is shown in Figure 6.2, where the pink square shows where the permanent magnet is and indicates the relatively higher magnetic field intensity, and the orange area indicates the relatively lower magnetic field intensity. The yellow area was not used for the cell study.

6.3.2 Cell Morphology

Cell morphology is an important indicator of cell functions. From Figure 6.3, it is hard to tell whether there is difference between each group. To determine the BMSC

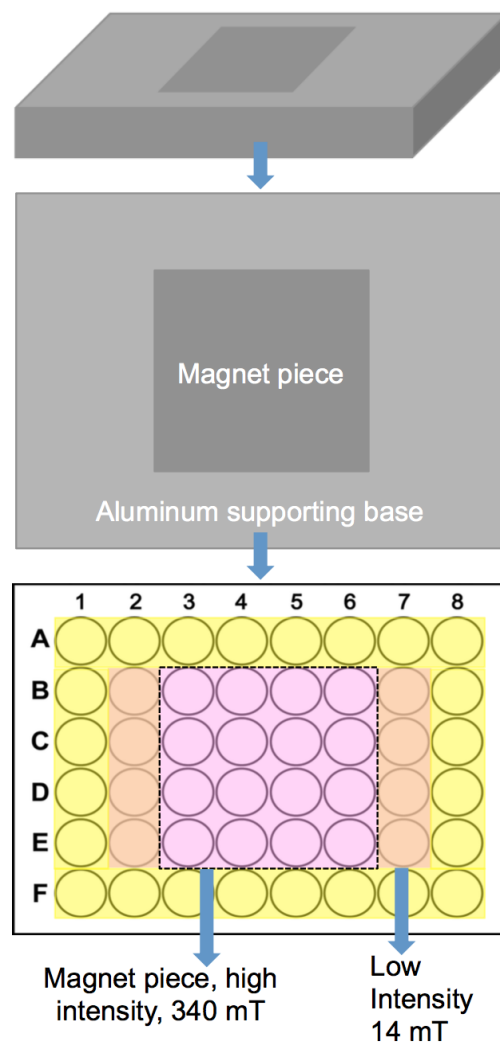


Figure 6.2: The magnetic field intensity distribution generated by the magnetic device.

morphology under high and low intensities of magnetic fields, we determined the Feret maximum diameter and Feret minimum diameter of each cell using ImageJ and calculated average BMSCs aspect ratio ($AS = \text{Feret maximum diameter} / \text{Feret minimum diameter}$).

For cell morphology, the ANOVA analysis showed that there was no statistically significant difference detected for the BMSCs cultured in the 16 wells under high magnetic intensity. Similarly, no statistically significant difference was detected for the BMSCs cultured in the 8 wells under low magnetic intensity. Additionally, no statistically significant difference was detected between the BMSCs cultured under high and low intensities of magnetic fields.

6.3.3 Cell Density

Figure 6.3 showed that we can tell that cells are near confluent in each group, we can clearly tell their nuclei and actin. For cell density, the ANOVA analysis showed that there was no statistically significant difference detected for the BMSCs cultured in the 16 wells under high magnetic field intensity. Similarly, no statistically significant difference was detected for the BMSCs cultured in the 8 wells under low magnetic field intensity (Figure 6.4).

Importantly, no statistically significant difference was detected between the BMSCs cultured under high and low intensities of magnetic fields, although the average BMSCs adhesion density was greater under high intensity magnetic field.

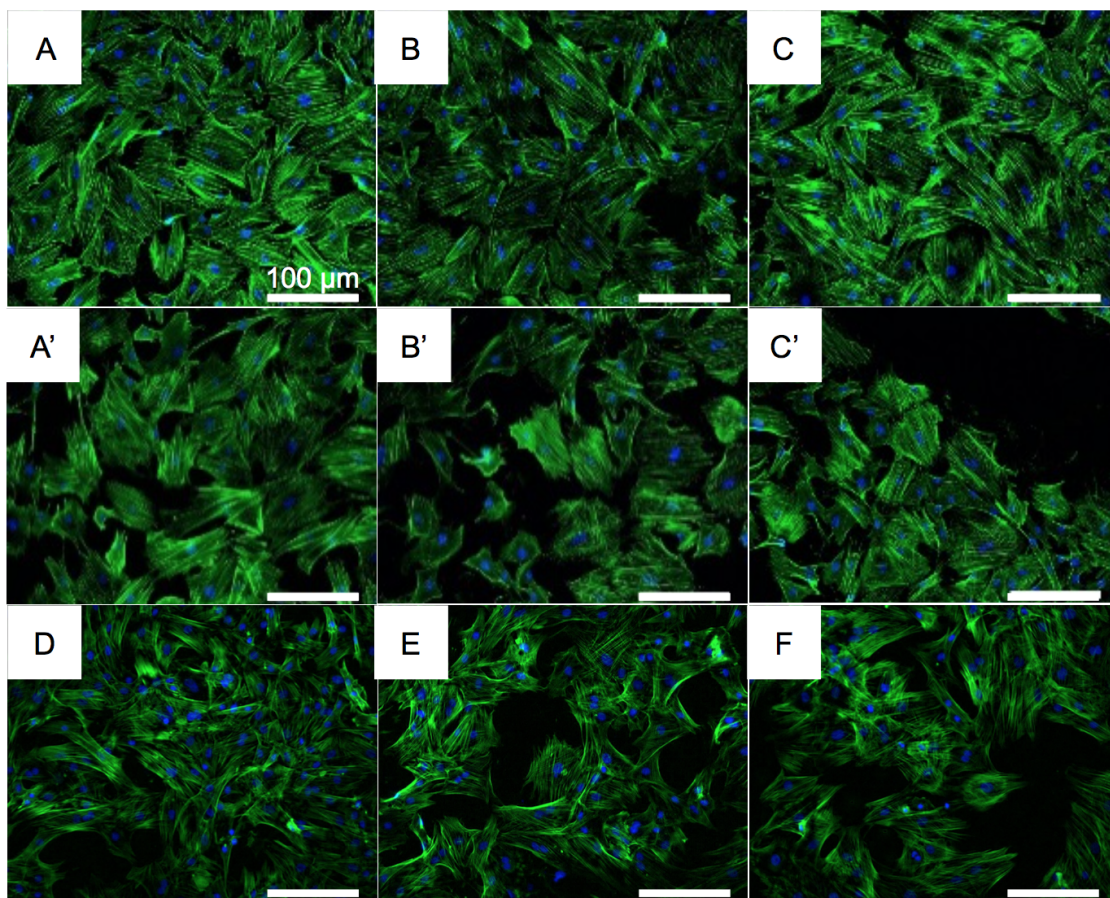


Figure 6.3: Fluorescence images of BMSCs under (A-C) high and (A'-C') low intensity magnetic field. Scale bar = 100 μm .

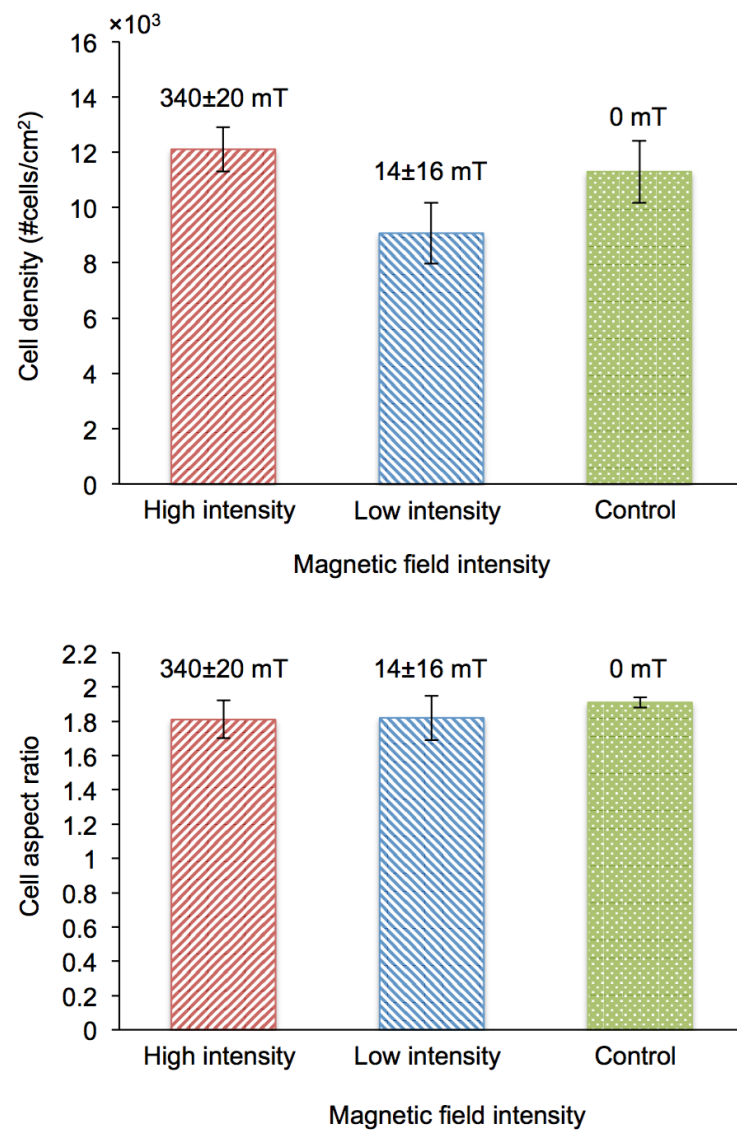


Figure 6.4: The average BMSCs density under high (340 mT) and low (14 mT) magnetic field intensity, respectively. The values are mean $\pm$ SEM. Shapiro test and Bartlett test showed that they had normal distribution.

6.3.4 Conclusions

The 72-hour culture results showed that the SMFs with a high intensity of 340 mT increased the average cell proliferation as compared with the field with a low intensity of 14 mT, but there was no statistically significant difference detected. The statistical analysis of the 72-hour culture results showed that the SMFs with a high intensity of 340 mT and low intensity of 14 mT had no influence on cell morphologies. Cells in high and low magnetic field intensity wells displayed a variety of morphologies and their aspect ratios were similar which indicated the cell morphology did not change with respect to magnetic field intensity.

Chapter 7

Influence of a moderate electromagnetic field on bone marrow-derived mesenchymal stem cell proliferation and differentiation behaviors

7.1 Introduction

The interaction of electromagnetic fields (EMFs) with living organisms is a rapidly growing research field. The future of this field spans from protecting human health from the increasing production of EMFs in modern society and the benefits of utilizing EMFs in new technologies such as magnetic orientation [42] and therapeutic use [52] in biomedical applications.

Current stem cell research in bone tissue engineering often relies on the use of growth factors to induce cell differentiation for therapeutic purposes [155] such as bone

morphogenetic proteins (BMP) [156] and transforming growth factor beta (TGF- β) [157] for osteogenesis and vascular endothelial growth factor (VEGF) for angiogenesis [158]. However, these growth factors are not only short-lived proteins but also expensive resources for stem cell research *in vitro*. Reports have indicated that EMFs have physiological effects on stem cell differentiation both *in vivo* and *in vitro*, including the callus formation in Spargue-Dawley mice [159] and up-regulation of type II collagen gene expression in human mesenchymal stem cells (hMSCs) [87]. Luo et al. showed that hMSCs exposed to a 50 Hz and 1.1 mT EMF for 30 minutes per day for 21 days *in vitro* had higher ALP activity, type I collagen expression and osteocalcin secretion compared to any other frequencies from 5 to 150 Hz [160]. Kang et al. showed that exposure to EMF with frequency of 30/45 Hz and intensity of 1 mT for 8 hours per day for 20 days helped increase osteogenic marker expression in human adipose-derived mesenchymal stem cells (hASCs) *in vitro* on day 7 compared to any other frequencies from 7.5 to 75 Hz [161]. Another study using EMF at 15 Hz and 2 mT increased hASCs chondrogenic marker and bone matrix gene expression after 7 days of culture *in vitro* [124]. However, few research reported the influence of EMF on the differentiation potential of bone marrow-derived mesenchymal stem cells (BMSCs), which are pluripotent stem cells that reside in bone marrow that can differentiate into multiple cell lineages depending on their environment [162]. BMSCs have long-term self-renewing capability that make them the ideal stem cells for bone tissue repair [163]. In light of the lower cost and higher sustainability of EMF usage compared to growth factors, we are further pursuing the use of EMFs as a potential alternative for inducing BMSC differentiation.

Due to their simplicity in design and fabrication, coils are commonly used to generate EMF in the field of bioelectromagnetics for testing the effects of EMF on biological functions and processes. A coil is a device usually consisting of multiple turns of copper wire wound around a frame. Variations of coil designs have been developed in the past that involve a combination of different parameters, such as coil shapes and sizes, number of coils, coil turn ratios, and coil positions relative to the center of the system. These frequently used coil designs named after the scientists who invented them are known as the Helmholtz 2 circular and 2 square, Lee-Whiting 4 circular, Merritt 3 and 4 square and Alldred & Scollar 4 square coil systems [164]. The coils in these designs are arranged coaxially to produce an area of uniform EMF intensity in the center of the system, and the size of this uniform area depends on the coil diameter. Thus, the desired EMF parameters that result from the different coil designs are taken into account when selecting the coil design for the required application.

Our research project involves designing and fabricating an EMF generating device to expose BMSCs to a low frequency (30 Hz) and low to medium intensity EMF (0.5 and 1 mT) to investigate the effects of EMF exposure on BMSC proliferation and differentiation behavior. In order to achieve this, COMSOL Multiphysics was used to simulate the six different coil configurations to determine a suitable coil design before fabricating the EMF generating device. An EMF shielding box was also fabricated to protect the environment outside the box from the influence of EMF generated by the device. BMSC functions were investigated during the three-week culture under EMF exposure.

7.2 Materials and Methods

7.2.1 COMSOL Simulation of Different Coil Designs

When conducting the COMSOL simulation, the type of physics to be tested was first selected, which in our case was the AC/DC-Magnetic Fields (mf) application. The geometry of the model to be simulated was then created based on each different coil design, with the end result being coaxial circular or square coils. The coil edges where the magnetic fields are simulated between were selected. The direction of current flow can be changed, thereby adjusting the magnetic field direction if desired. For our application, however, the direction of the magnetic field is irrelevant. Finally, the appropriate size and settings for mesh generation are inputted, the study is computed, and plots are then generated to aid in visualizing and analyzing the data obtained. To achieve an EMF intensity of 1 mT in our experiment, only a current of 500 mA was used. The diameter of the coil was set to 14.88 cm, which provides an area of uniform EMF intensity to encompass the entire well plate, while still minimizing the size of the overall coil system. A default conductivity of 6.00×10^7 S/m was used for the copper wire at approximately room temperature. Also, using the coil diameter chosen above, the coil positions were determined from the coil spacing from center of system relations for each coil design.

7.2.2 Fabrication of EMF Generating Device

After determining the coil design most suitable for our project, we then needed to determine how we wanted the EMF generating device fabricated. Since there was no coil

system in the market that met our required specifications, a combination of SolidWorks modeling and 3D printing was chosen for simple yet precise manufacturing as well as convenience in modifying and reproducing the device. In addition, the 3D printed components are made out of ABS plastic, a light and strong material, yet easy to be sterilized for use in the cell culture incubator, to minimize fabrication cost and increase maneuverability of the device once fully constructed. The different components of our EMF generating device, which consisted of a base, 4 coil frames, 2 handles, a table, and 3 horizontal connectors, were first modeled in SolidWorks individually as part files before checking for interferences when assembled in an assembly file. The base of the device serves to hold the coil frames in place. The 4 coil frames with copper wire wound around based on the predetermined number of wire turns per coil make up the Lee-Whiting 4 circular coil system. The 2 handles on either side of the base allow the device to be picked up and carried for easy transport. A table is placed in the center of the coil system where the well plate rests on and is at a height in the middle of the system where area of uniform EMF intensity is maximum. The 3 horizontal connectors align the 4 coil frames at the top to ensure each coil is parallel with one another. The individual SolidWorks part files are then saved as STL files for the 3D printer to read and print. Assembling the EMF generating device involved connecting the printed components via slot insertions or hole-rod insertions.

7.2.3 Fabrication of EMF Shielding Box

EMF shielding box was made of MagnetShield plate (Less EMF Inc., Latham, NY) and designed to be a 30×30 cm five-sided box with an open on the top. To achieve enough shielding effectiveness, each side was triple-layered and the edges of the shielding plates were covered with soft Joint Shield (Less EMF Inc., Latham, NY) to prevent EMF from leaking out and affect outside areas.

7.2.4 Testing of EMF Generating Device and Shielding Box

The EMF generating device and shielding box was tested with a programmable DC power supply and a gaussmeter (Lakeshore). The EMF generating device was placed into the shielding box and connected with the DC power supply that drives the current flow through the coil system via lead cords plugged into metal sockets attached near the base of the coil frames. The ends of the copper wires wound around the coil frames are bent into hooks that make contact with the metal sockets, establishing the closed circuitry necessary for current flow. With the current set to 500 mA and 1 A, the EMF intensities inside the coils in the shielding box and outside the shielding box were all tested with the gaussmeter. The tested EMF intensities were compared with COMSOL simulation results, and the EMF generating device was tested to have no heating effects.

7.2.5 Cell Culture in the EMF Generating Device and Shielding Box

Bone marrow-derived mesenchymal stem cells (BMSCs) were cultured in T75 flask until confluent. EMF generating device and shielding box were sprayed and wiped with 70% ethanol, respectively, and placed in laminar flow hood afterwards for overnight UV-sterilization. The device and shielding box were then placed in cell culture incubator 24 hours ahead of cell seeding in order to reach temperature stabilization.

Confluent flask of BMSCs was washed with PBS to remove dead cells and detached with trypsin-EDTA (#25200-056, Life Technologies, Carlsbad, CA). Fresh media was added to the vial to make cell suspension and the cells were count manually with a hemocytometer (#1475, Reichert Bright-Line, Hausser Scientific, Horsham, PA) under microscope. Cell seeding density was set to 20,000 cells/cm² based on previous publications [ref], in order to reach confluency after 24 hours. Cells were seeded in 12-well chamber slides (#81201, ibidi, Madison, WI) in triplicate groups and cultured in standard condition (i.e., 37 °C, 5% CO₂/95% air, humidified environment) without EMF for 24 hours to get initial density. After 24 hours, the EMF device was connected with external power supply that could provide 30 Hz and 500 mA triangle wave. Half of the groups were placed on top of the platform inside EMF generating device for 21-day culture, and another half without EMF for 21-day culture in comparison. The control groups were placed in the same incubator under the EMF shielding box with a distance of 25 cm, which position was tested to be not affected by the EMF generating device.

7.2.6 BMSC Proliferation and Morphology Characterization

BMSCs were fixed and stained at 24 hours prior to the application of external EMF, and 24 hours, 1 week, 2 weeks and 3 weeks after the application of external EMF to examine their proliferation and morphology. Adherent BMSCs were fixed with 4% paraformaldehyde (PFA; #15714-S, Electron Microscopy Science, Hatfield, PA) solution, stained with Alexa Fluor 488 solution (#A12379, Life Technologies, Carlsbad, CA) for F-actin visualization and Hoechst 33342 solution (#H3570, Life Technologies, Carlsbad, CA) for nucleus visualization. The cells were then imaged using an inverted fluorescence microscope (Nikon Ti-S eclipse, Nikon, Japan). For comparison, the cell proliferation was also quantified with a Quick Cell Proliferation Colorimetric Assay Kit Plus (#K302-500, BioVision, Milpitas, CA). At each time point, 10 μ L WST reagent was added to each well and incubated with cells for 3 hours in standard cell culture conditions, and 10 μ L stop solution was added to stop the reaction. The cell culture media together with WST reagent and stop solution was then transferred to a 96-well plate for absorbance reading at 460 nm using a micro-plate reader (Model Infinite M200 Pro, Tecan, Switzerland). Bared media together with WST reagent and stop solution was used as reference group.

7.2.7 Cell Calcium, GAG and Collagen Deposition Characterization

Calcium deposition was stained with Alizarin Red S staining dye (LC10590, LabChem). Cell culture media was removed at week 1, 2 and 3 time point, and the cells were carefully washed one time with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$, fixed with 4% PFA solution and stained

with Alizarin Red S solution for 45 minutes in dark. Then the cells were washed with DI water for five times.

Glycosaminoglycan (GAG) was stained with Alcian Blue 8G staining dye (TMS-010-C, Millipore). Cell culture media was removed at week 1, 2 and 3 time point, and the cells were carefully washed twice with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$, fixed with 4% PFA solution and stained with Alcian Blue solution for 45 minutes in dark. Then the cells were washed with DI water for three times.

Collagen deposition was stained with Sirius Red staining dye (#26357-02, Electron Microscopy Science, Hatfield, PA). Cell culture media was removed at week 1, 2 and 3 time point, and the cells were carefully washed one time with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$, fixed with 4% PFA solution and stained with Sirius Red solution for 1 hour in dark. Then the cells were washed with 0.01 M HCl for five times to remove unbound stain.

The silicon chambers of the slides stained with Alizarin Red S, Alcian Blue and Sirius Red were then removed to allow the adherent cells dry out, mount with mounting media (#4112, Thermo Fisher Scientific, Inc., Waltham, MA) and protected with cover slides. The slides were observed and imaged in the bright field of the inverted fluorescence microscope (Nikon Ti-S eclipse, Nikon, Japan).

7.2.8 Cell Differentiation Colorimetric Characterization

BMSCs were lysed with freeze-thaw method. At week 1, 2 and 3, cell culture media was removed and cells were rinsed with PBS and DI water each for one time. Same

volume of DI water was added to each group, repeatedly froze in -20 °C freezer for 3 hours and then thaw at room temperature for 4 cycles. The cell lysates were then examined with quantification methods of calcium deposition, ALP activity, protein content and collagen secretion.

QuantiChromTM Calcium Assay Kit (#DICA-500, BioAssay Systems) was used for the quantification of calcium ions in the study. Each reagent was equilibrated to room temperature prior to use. 5 μ L diluted standards and cell lysates were transferred into wells of a clear-bottom 96 well plate, and 200 μ L working reagent was added to each well and tap lightly to mix. The mixture was incubated at room temperature for 3 minutes and the optical density was read at 612 nm using the micro-plate reader. Calcium ion concentration was calculated from the subtraction of blank controls.

QuantiChromTM Alkaline Phosphatase Assay Kit (#DALP-250, BioAssay Systems) was used for the quantification of ALP activities in the study. Each reagent was equilibrated to room temperature prior to use. 200 μ L DI water and calibrator was pipetted into separate wells of a 96-well plate as control groups. 50 μ L cell lysates from each group per time point were pipetted into 96-well plate and 150 μ L working solution was added to each well to mix with samples. The mixture was tapped briefly and absorbance was read at $t = 0$ and $t = 4$ minutes at wavelength of 405 nm. ALP activity was calculated with the equation provided by the company.

Total protein content was quantified with a Micro BCA Protein Assay Kit (#23235, Thermo Fisher Scientific, Inc., Waltham, MA). 150 μ L of series standard protein solution

and unknown cell lysates were pipetted into 96-well plates in triplicate, and 150 μ L of working reagent was added to each well and the plate was mixed by shaking for 30 seconds. The plate was covered using a sealing tape (#15036, Thermo Fisher Scientific, Inc., Waltham, MA) and incubated at 37 °C for 2 hours. After cooled to room temperature, the absorbance of all the samples was read at 562 nm with a micro-plate reader. A standard curve was plotted based on standard protein concentrations and the total protein concentration of each sample was calculated with the standard curve and presented in μ g/mL.

Sirius Red staining dye was also used for the quantification of collagen secretion. Standard collagen solution was prepared by dissolving lyophilized bovine type I collagen (#1200-02, SouthernBiotech) into DI water and serially diluted into different concentrations. 50 μ L of standard collagen solution and cell lysates were pipetted into a 96-well plate and dried in an incu-shaker (Incu-shaker Mini, Benchmark Scientific, Edison, NJ) for 2 days at 37 °C and 100 rpm. Then 50 μ L of Sirius Red staining dye was added to each well and incubated at room temperature for 1 hour. After the dye was removed, all wells were rinsed with 0.01 M HCl for five times to remove unbound stain and the stain was collected by washing with 0.1 M NaOH for 5 minutes. The collected stain was then transferred to another 96-well plate and the absorbance was read in the micro-plate reader at 540 nm. A standard curve was plotted and collagen content of the samples was calculated.

7.2.9 Statistical Analysis

Experiments data were analyzed in R using one-way analysis of variance (ANOVA) followed by the Tukey highest significant difference post hoc test. Statistical significant difference was considered at $p < 0.05$.

7.3 Results

7.3.1 COMSOL Simulation of Different Coil Designs

Results showed that while both the Lee-Whiting 4 circular coil system and Merritt 4 square coil system were able to achieve the same highest field intensity in the center of the system, the Lee-Whiting design required less spacing between the coils and fewer turns per coil, allowing for a more efficient use of space and materials (Figure 7.1). In conclusion, the Lee-Whiting 4 circular coil system can provide the desired EMF intensity, uniform area and coil spacing for the design of EMF generating device, which ensured the uniform exposure of sufficient amount of chamber slides to the EMF generated by the device while the device can still fit in the incubator for the future test of cell functions.

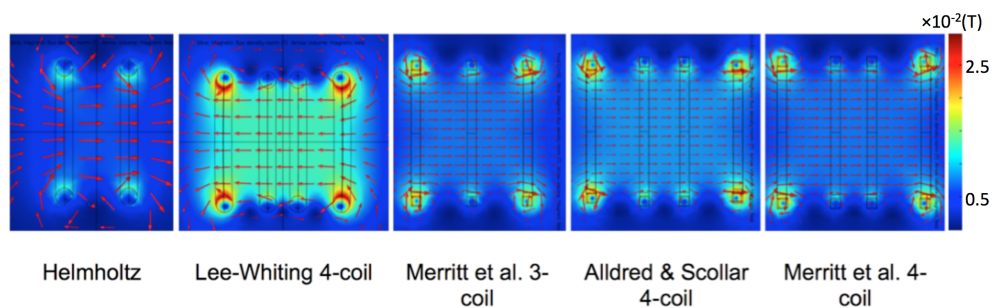


Figure 7.1: COMSOL simulation results of different coil designs.

7.3.2 Testing of EMF Generating Device and Shielding Box

The 3D-printed and assembled EMF generating device is shown in Figure 7.2. From the gaussmeter testing results, the real EMF intensity generated at the center of the device was 11.1 ± 0.7 gauss (1.11 ± 0.07 mT) when the current was 500 mA and 20.2 ± 0.6 gauss (2.02 ± 0.06 mT) when the current was 1 A, which were consistent with the COMSOL simulation results of 10 gauss (1 mT) at 500 mA and 20 gauss (2 mT) at 1 A, respectively. The table of the simulation comparison also showed that EMF generated by single 80-turn coil and single 180-turn coil were consistently match with the COMSOL simulation results.

When apply the shielding box, the field intensity at the center of the device was not affected while the field intensity outside the shielding box was reduced significantly. When the current was set to 1 A, the EMF intensity at 10 cm away from the outside of the three-layered shielding box was 0.4 gauss, which was 0.04 mT and in the range of geomagnetic field. The test results showed that the EMF generating device could provide the field intensity as needed and consistent with the COMSOL simulation results, and the shielding box could shielding the inside field from affecting the outside environment.

7.3.3 Cell Proliferation Characterization

Cells reached confluency after 24 hours of culture prior to EMF exposure. Fluorescence images (Figure 7.3) showed that cell density and morphology was similar between each groups after 24 hours and 1 week of culture with and without EMF exposure. However, starting week 2, cell aggregates were observed in all groups, including EMF + OM, OM,

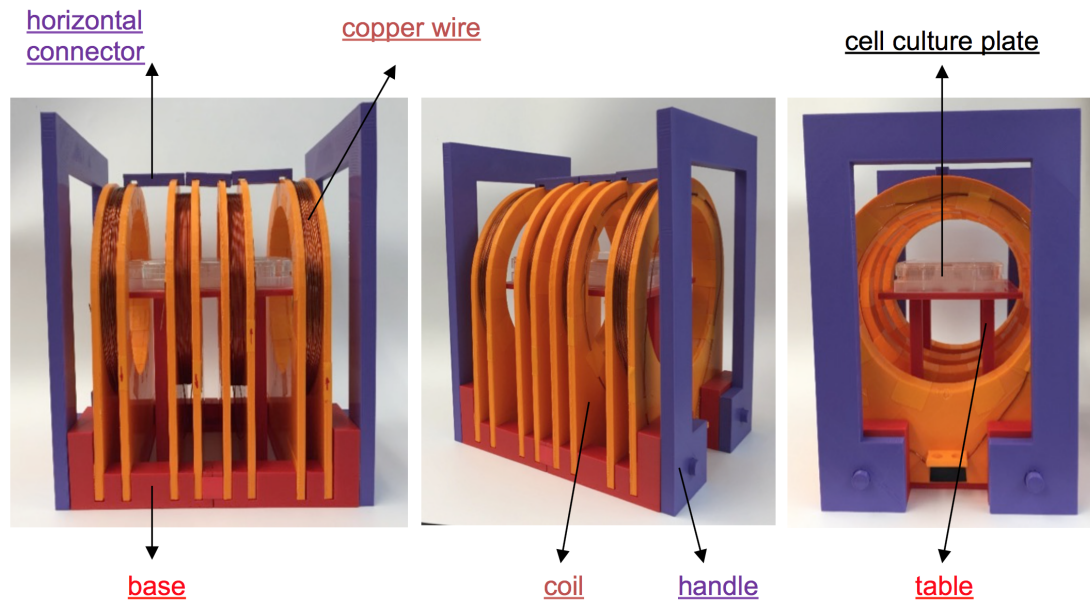


Figure 7.2: Photos of EMF generating device.

EMF + BM and BM groups, and became bigger at week 3. Those aggregates in EMF + OM and OM groups were irregular-shaped clumps, the aggregation of cells were pockets of layer-by-layer cell nodules with cells sheets around them. The center of the aggregates was too thick to be imaged clearly with a single focus. While those aggregates in EMF + BM and BM groups were more regular round-shaped, with brighter color on the edge, and the aggregates were smaller than those in the EMF + OM and OM groups.

The WST proliferation assay showed that the cell density in each group had a decreasing trend over time but there was no significant changes, and the cell density had no significant difference between groups. This result was consistent with the fluorescence staining result (Figure 7.4).

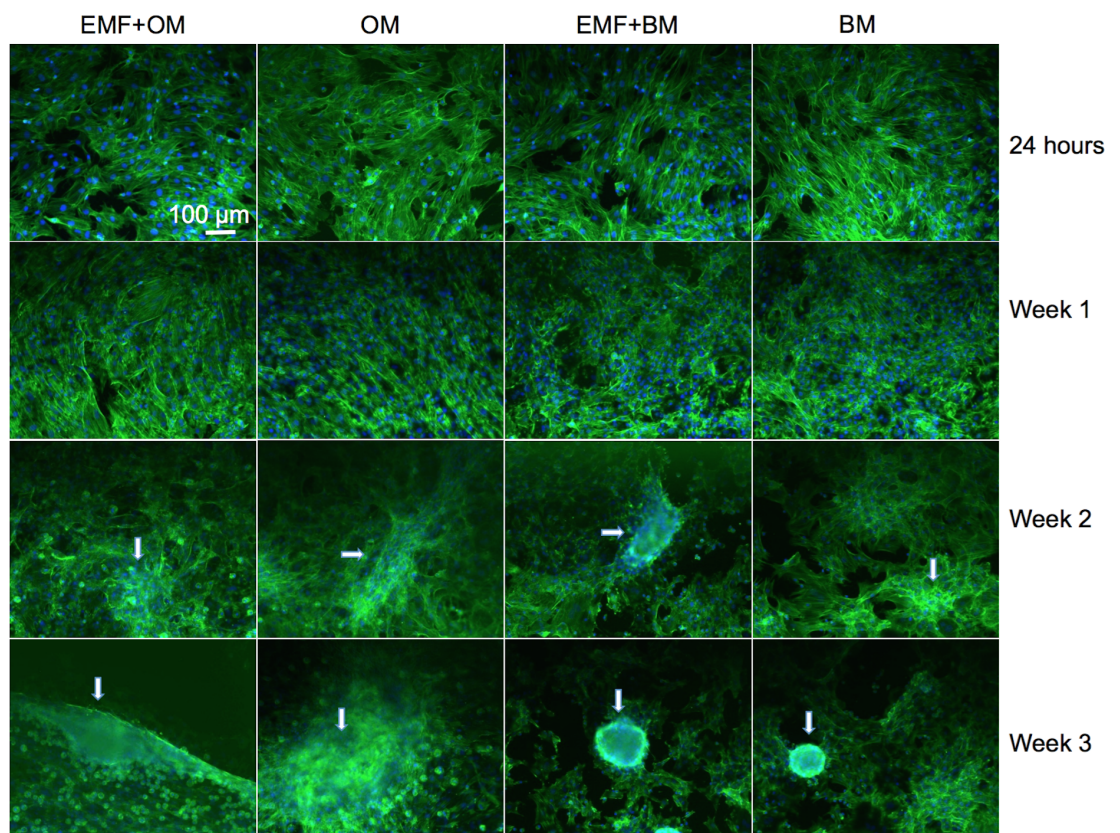


Figure 7.3: Fluorescence imaging. White arrow shows cell aggregates.

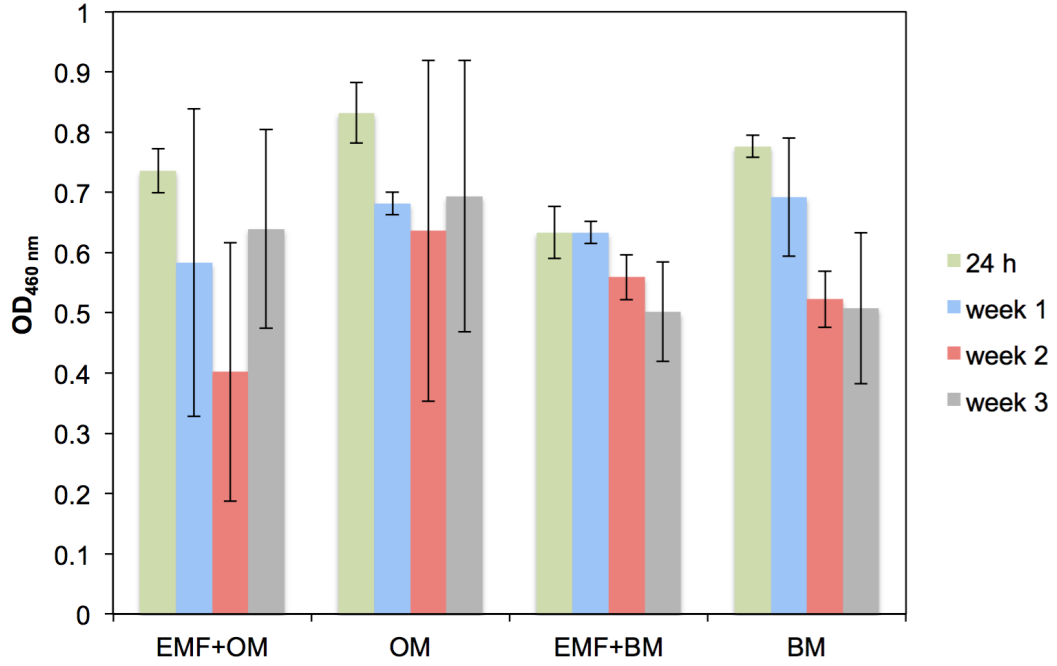


Figure 7.4: WST cell proliferation assay.

7.3.4 Cell Differentiation Staining

The BMSC differentiated into osteogenic lineage after 2 weeks of culture in osteogenic induction media was confirmed by presence of extracellular mineralization as evident on Alizarin Red S staining shown in Figure 7.5. After Alizarin Red S staining, mineralized extracellular deposits were observed at week 2 and more found at week 3. At week 1 and week 2, there are more cell aggregates in the EMF groups compared to OM or BM alone groups. Those aggregates were stained light red, it might be small amount of extracellular mineral deposit or due to the layer-by-layer cell overlapping. However, at week 3, the EMF + OM group and OM group had significant amount of bright red staining, indicating calcium deposition, and the EMF + OM group had more red areas compared to

the OM group. EMF + BM and BM groups did not have bright red staining, but the EMF + BM group had more cell aggregates than BM group, indicating that the EMF might have positive influence on BMSC calcium deposition.

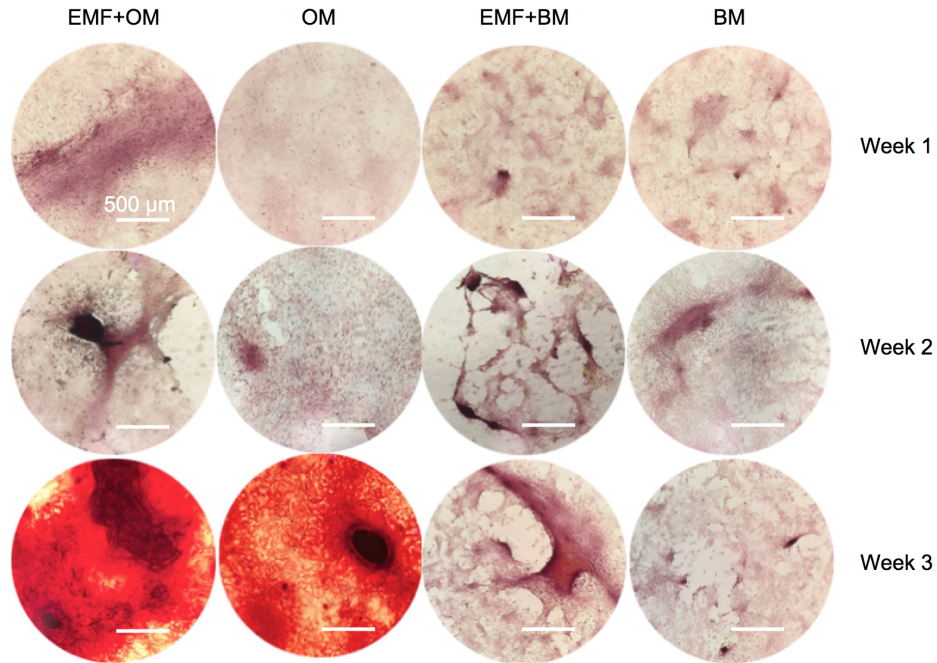


Figure 7.5: Alizarin Res S staining.

Alcian Blue staining showed increasing amount of GAGs secretion from week 1 to week 3 in all groups, and the staining were much bigger in OM groups than in BM groups (Figure 7.6). However, there were no significant difference between EMF + OM and OM groups and no significant difference between EMF + BM and BM groups. The dark blue staining areas were also consistent with the cell aggregate areas, and with the Alcian Blue staining, the center of the aggregate was too thick to be translucent.

Sirius Red staining showed increasing amount of collagen deposition from week 1 to week 3 in all groups. The staining was similar in EMF + OM and OM groups, and was closely connected with each other (Figure 7.7). The staining was also similar in EMF + BM and BM groups, although those areas were also connected with each other, they were loosely connected and much smaller than those in the OM groups indicating less collagen deposition. Osteogenic media was able to induce collagen secretion compared to control groups.

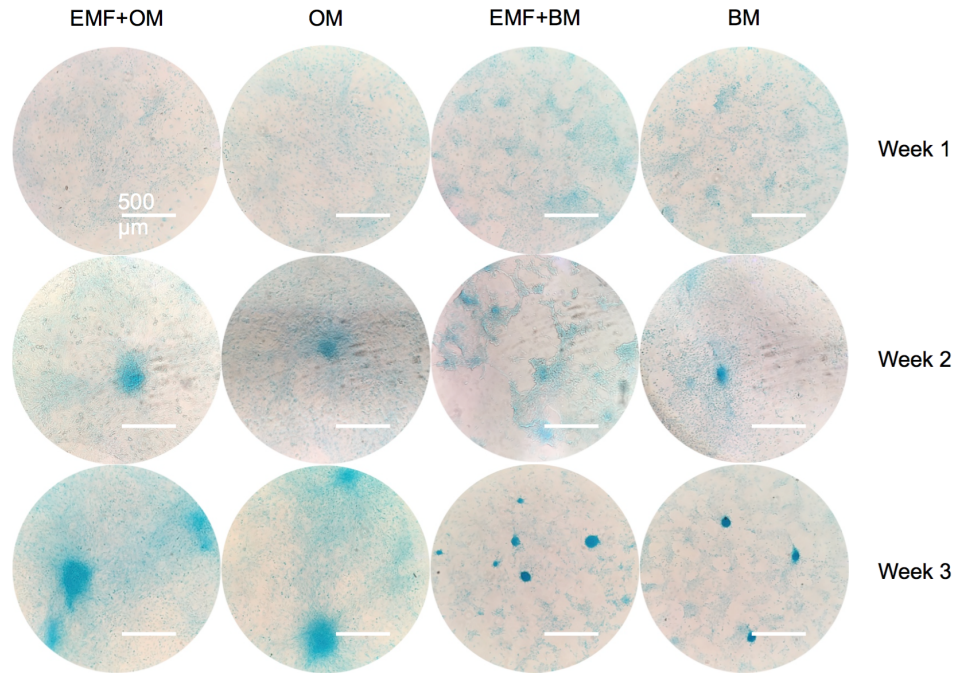


Figure 7.6: Alcian Blue staining.

7.3.5 Cell Differentiation Colorimetric Characterization

Calcium ion concentration was low and similar in all four groups at week 1, and at week 2, there was a significant increase in OM groups alone (Figure 7.8). The calcium ion

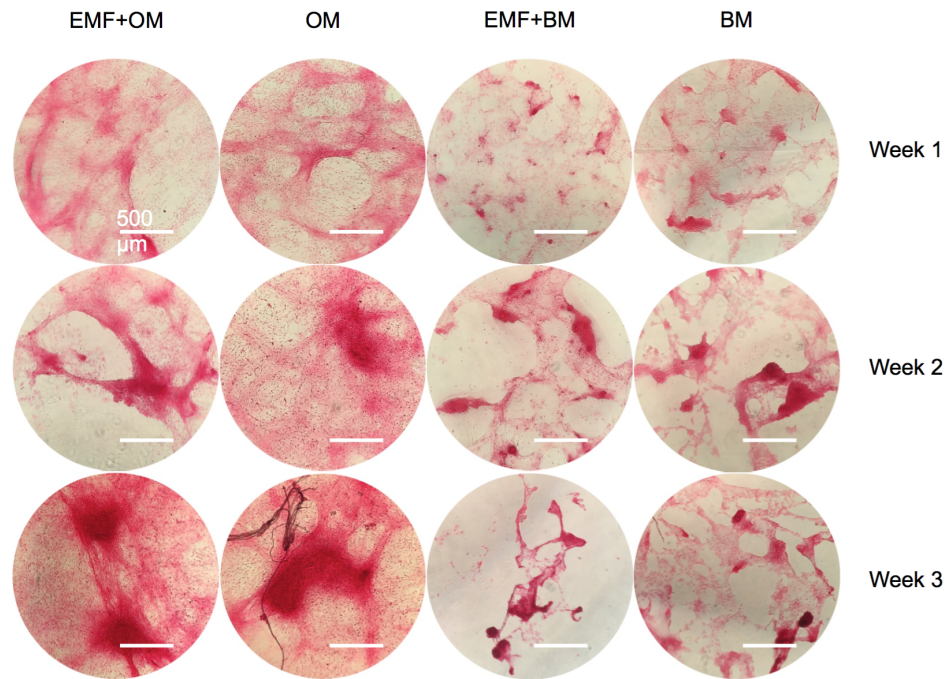


Figure 7.7: Sirius Red staining.

concentration in EMF + OM and OM groups was much higher at week 3, reached 23 and 27 mg/L, respectively. The results showed that there were more free calcium ion in the OM groups compared to BM groups. More free calcium ion might indicate less calcium mineral deposition, which was consistent with the Alizarin Res S staining results. OM group had higher calcium ion concentration in week 2 and week 3 compared to EMF + OM group, consistent with lower calcium deposition. Both EMF + OM and OM group has higher calcium ion concentration in week 3 than in week 2 might indicate that cells were more actively intake free calcium ion from media but were not fast enough to deposit all free ions into mineral.

ALP activities in the OM groups increased over time while decreased over time in the BM groups (Figure 7.8). ALP activities in week 1 in OM groups were lower than in BM groups, however, they were higher at week 2 and week 3. Compare EMF + OM group with OM group, their ALP activity was similar at each time point, and so does EMF + BM and BM group. This might indicate that EMF did not have significant influence on the ALP activities of BMSCs. However, with osteogenic media, cell activity was much increased over time, indicating active differentiation, but in the BM groups, cells were unable to maintain their activity.

Total protein concentration in the OM groups increased over time while increased and then decreased over time in the BM groups (Figure 7.9). Total protein concentration in BM groups were higher than OM groups at week 1 and week 2, however, it was lower at week 3 compared to OM groups. The changes of protein concentrations in OM groups showed that with the osteogenic media, BMSC was in active osteogenic differentiation and secret increasing amount of protein along with time. At the mean time, the activity of BMSC in BM groups was not able to maintain for a long time and the osteogenic differentiation was not induced. However, there was no significant difference of total protein concentration among all groups. Besides, this total protein assay could only detect protein but not able to differentiate various type of proteins, which need further confirmation.

From the collagen concentration diagram, collagen deposition increased in all four groups over time (Figure 7.9). The collagen concentration was similar in all four groups

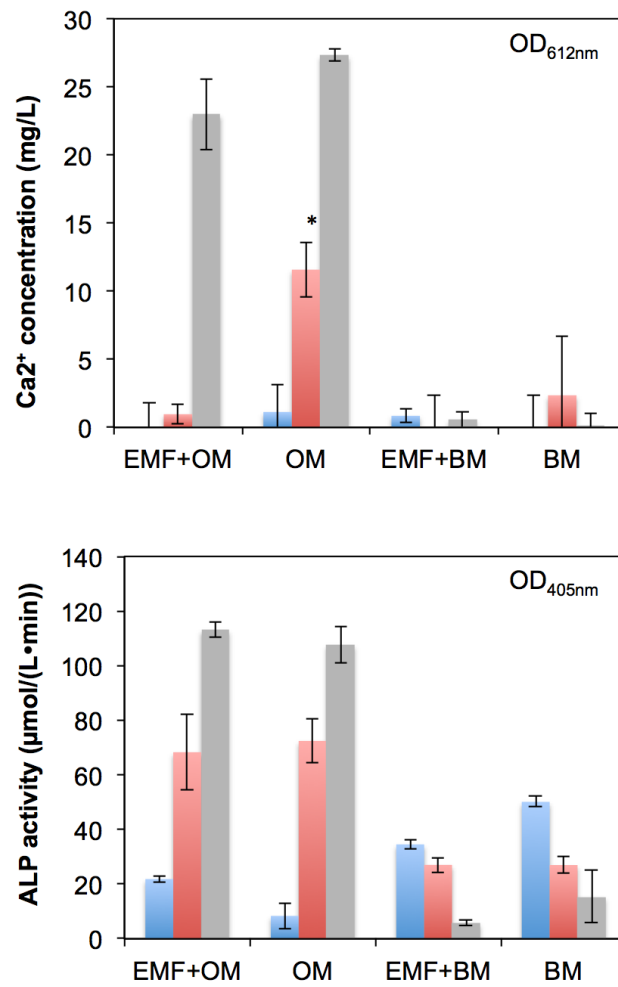


Figure 7.8: Calcium ion concentration and ALP activity quantification.

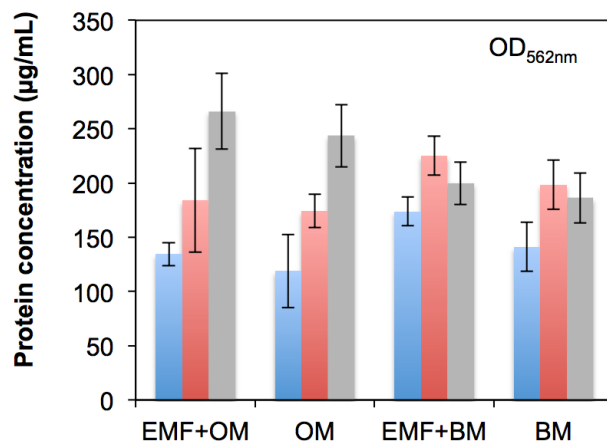
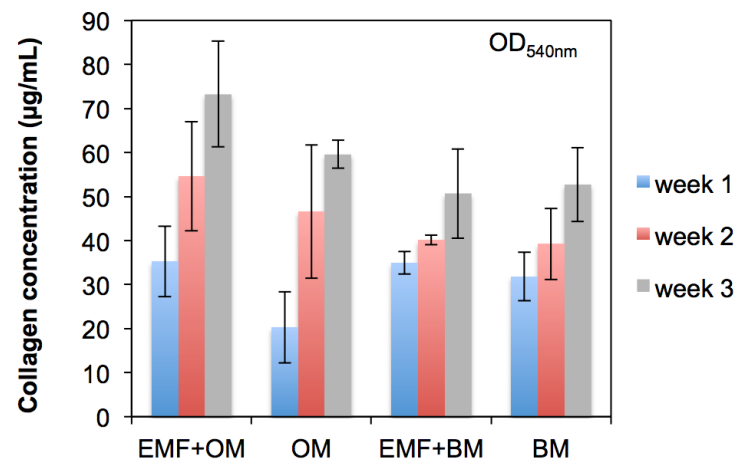


Figure 7.9: Collage and total protein concentration quantification.

at week 1 and week 2, and it was slightly higher in the OM groups compared to the BM groups. This result was consistent with the Alcian Blue staining results.

7.4 Discussion

7.4.1 COMSOL Simulation and EMF Device Fabrication

COMSOL simulation of all the six different classic coil designs showed that Lee-Whiting 4 circular coil design had relative large uniform EMF area at the same EMF intensity with less wire turns per coil, and the coil spacing would allow a better fit into the incubator. The simulation result was consistent with the calculation of electromagnetic fields published in 1992 [164]. There was no advanced software like COMSOL Multiphysics in 1992 that could help simulate single or coupled physics without the need of device-based experiments. Scientists at that time could only use complicated equations to calculate physics and run experiment to verify their calculation. The consistent results obtained from COMSOL simulation not only proved the great work of previous scientists, but also provided ideas to us on which coil design that we should choose.

Since the BMSCs are cultured in a well plate and placed in the center of the coil system, a large area of uniform EMF intensity is important to ensure the entire cell culture plate is exposed to EMF of the same intensity. This means that our different study groups in the separate wells of the plate are tested under the same conditions, which allows for proper comparison between study groups. The two coil designs that achieved the largest areas of uniform EMF intensity in our simulations are the Merritt 4 square and the Lee-

Whiting 4 circular coil systems, which were the two options we mainly considered for design selection. The Lee-Whiting design has tighter spacing between coils than the Merritt design, which decreases the overall size of the coil system, while maintaining nearly the same area of uniform EMF intensity. Since the coil system will be placed in an incubator to allow for optimal growth temperature while the BMSCs are exposed to EMF, a smaller coil system provides more efficient space usage. Furthermore, the Lee-Whiting design requires more than two times fewer wire turns per coil, reducing the necessary coil thickness and amount of copper wire. Therefore, the Lee-Whiting design is more cost effective by requiring less material which ultimately reduces the cost of fabrication. Finally, although the Lee-Whiting design produces more than two times less EMF intensity in the center of system when compared to that of the Merritt design, this is the least deciding factor since our application of testing the biological effects of EMF exposure on cells only requires a low EMF intensity of 1 mT. Furthermore, if a higher EMF intensity ever became necessary in our experiments, the input current could simply be increased accordingly.

3D printing was chosen for the fabrication of coil frames not only because of its easy to design and manufacture, but also due to its flexibility compared to the pre-made commercial coil frames. ABS plus has enough strength to hold multi-turn wires, able to be ethanol-sterilized and UV-sterilized without forming free radicals that could harm cells. Besides, ABS plus is very cost-effective. The assembled EMF generating device and shielding box was tested and the test results showed that the device could generate EMF

as needed and consistent with the COMSOL simulation results, and the shielding box was able to prevent the EMF from leaking out thus protect the outside environment.

7.4.2 BMSC Proliferation and Differentiation Behavior

WST quantification showed that cell density did not change significantly over time in each group, which was consistent with the observed cells density in fluorescence images. Cell morphology did not change within the first week in each group, however, there were cell aggregates formed in each group at week 2 time point, which made it difficult to visualize individual cell morphology. Thus, different quantification methods were used to characterize BMSC differentiation.

Alizarin Red S staining showed the presence of calcium minerals, and those in BM groups formed slower than in OM groups. The free calcium ion quantification method showed less free calcium ion in the EMF + OM group compared to the OM group, which was consistent with the Alizarin Red S staining showing more calcium deposition in the EMF + OM group. Osteogenic media could induce the osteogenic differentiation of cells, making them more actively intake calcium ions from media and deposit into minerals in order to readily form bone modules. More free calcium ion might indicate less calcium mineral formation, which was proved by the staining results. While more free calcium ion in the OM groups compared to BM groups proved the ability of inducing calcium ion intake of osteogenic media.

Collagen secretion and ALP activity was higher in OM groups than in BM groups, but there was no significant difference between each groups. Total protein concentration kept increasing at week 3 in OM groups while decreased in BM groups, which might result from osteogenesis. However, the type of protein in each group was not able to be differentiated by total protein assay, thus individual protein detection assay should be used in future studies.

7.5 Conclusions

This study showed that EMF helped increase calcium deposition in osteogenic media groups while there was no significant difference of protein and collagen secretion and ALP activities between EMF and no EMF groups cultured with osteogenic media or base media. However, the supplementation with Dexamethasone, beta-glycerophosphate and L-ascorbic acid helped increase all cell differentiation activities including calcium deposition, protein and collagen secretion and ALP activities.

7.6 Acknowledgements

We would like to thank Dr. Nissim Amos for his kind help with the use of gaussmeter. We thank Junchao Wang and Dr. William Grover for their kind help on the final coding of Arduino Uno R3. We thank Central Facility for Advanced Microscopy (CFAMM) at the University of California, Riverside, for the use of SEM and Ilkeun Lee and Dr. Krassimir Bozhilov for the training on SEM.

Chapter 8

Porous Magnetic Poly(glycerol sebacate) Nanocomposites Increased the Differentiation Potential of Bone Marrow-Derived Mesenchymal Stem Cells

8.1 Introduction

In adults, bones are remodeled and rebuilt through a process called bone turnover. It results in the removal of old bones and the replacement by new bones thus ensures the repair of any micro-damages and enables the regulation of mineral homeostasis [1, 2, 3]. This process is seen in healthy bone and bone formation keeps in pace with bone destruction. When bone destruction is greater or faster than bone formation, osteoporosis will happen [165], which will lead to low bone mass, bone fragility and an increased risk of fracture of the hip, spine and wrist. The National Osteoporosis Foundation (NOF) and National

Institutes of Health (NIH) reported that by 2013, there were 9 million adults in the U.S. suffering from osteoporosis, which is approximately 3% of the population. 43 million adults in the U.S. have low bone mass and 1.5 million people suffer fractures each year in the U.S. because of osteoporosis. And also, by 2020 half of all Americans over 50 will have weak bones.

Current major treatment methods for osteoporosis are drugs. The goal of treatment is the reduction of fractures which can be achieved by decreasing bone resorption and/or by increasing bone formation [4, 5]. The predominant FDA approved drug for osteoporosis therapy is bisphosphonate and a new competitor, denosumab, which can inhibit bone resorption through the inhibition of RANKL activity on the surface of osteoclasts. Bisphosphonate is the easiest to use compared to any other drugs. It has been shown to increase bone mineral density and has a terminal half-life of 10 years in bone [5, 6]. However, the long-term treatment will cause osteonecrosis of the jaw and atypical femoral subtrochanteric fracture [4, 5]. Denosumab can reduce fractures efficiently but it can also affect immune system because RANKL is expressed not only on bone cells but also by the immune system [5]. Other drugs such as teriparatide increases bone mineral density and new bone formation with a successful rate as high as 60% [7], however, not all patients respond to this drug and it is limited to a 2-year-cycle of treatment.

Stem cells have clonogenic and self-renewing capabilities and able to differentiate into multiple cell lineages [8], thus stem cell therapy has attracted more and more attention [6, 7, 9]. Stem cell therapy not only can reduce susceptibility of fractures and augment lost

mineral density, but also increase or restore the function of resident stem cells when compared to drug treatment [7]. But it also faces many problems for example the uncertainty of stem cell fate and biodistribution following cell transplantation. It has been reported that mesenchymal stem cells do not spontaneously engraft and differentiate upon infusion [7].

Naturally, stem cell differentiation occurs in a specific environment which includes growth factors, inner stimulation, molecular interactions and so on, thus requires complex and expensive sources for the stem cell to respond and differentiate into the cell types that is needed for bone repair [155]. Thus, if we can apply some exterior stimulation to help induce cell differentiation on biocompatible and biodegradable tissue engineering scaffolds *in vitro* then transplant them into bone tissue, we can control bone repair more efficiently and effectively.

Electromagnetic field (EMF) stimulation has been proved to have influence on stem cell differentiation. The energy exchange in the magnetic field can interact with cell molecules, which changes their natural kinetics or structures and in consequence, induces their function in cell differentiation. These effects depend on the frequency, density, and uniformity of the magnetic field, while exposure time, cell type, culture environment and other factors may also have influence on final impacts [28].

Considering the lower cost and higher sustainability of a EMF generating device compared to growth factors, we are further pursuing the use of magnetic fields as a potential alternative for inducing stem cell differentiation in couple with the magnetic nanocomposite

scaffold synthesized in lab. Furthermore, an EMF generating device can potentially generate both static magnetic field and time-varying magnetic field by the controlling of frequency, and achieve different intensities with different current input.

This project focused on investigating the ability of magnetic nanocomposite scaffolds coupled with magnetic fields to induce BMSCs differentiation for bone repair. In the following, I will describe how I propose to make magnetic composite scaffolds, how to design exterior magnetic fields and how to conduct cell study for the induction of BMSCs differentiation.

8.2 Materials and Methods

8.2.1 Synthesis and Characterization of Porous Magnetic PGS Nanocomposites

Synthesis of Porous PGS Nanocomposites

The synthesis of magnetic PGS nanocomposites was described in previous chapters. Briefly, PGS resin was purchased from Secant Medical (Secant Medical, Inc. Telford, PA) and processed to porous scaffolds use salt leaching method adapted from Wang et al. [141]. Briefly, NaCl salt crystals (Sigma) was ground and sieved to the size of 125-150 μm and added to a PFA mold with size of 8.2 $\times$ 3.7 $\times$ 1.8 cm. 2 mL of 100% ethanol was added subsequently to the mold with gently shaking to achieve homogeneous dispersion of NaCl crystals in the mold. The whole setup was air dried for 5 hours to produce a flat salt template with a thickness of 200 μm . The salt template was then incubated in an incu-shaker

(#H1000-M, Incu-shaker mini, Benchmark Scientific, Inc. South Plainfield, NJ) with relative humidity of 88-95% for 2-3 hours to fuse the NaCl crystals. Afterwards, it was dried in a vacuum oven under 60 °C and 10 torr absolute pressure for 12-18 hours. PGS resin was dissolved in 100% ethanol to a concentration of 513.9 mg/mL, and then different amount of MNPs was added to PGS solution for the preparation of PGS nanocomposites with 0, 1 and 10 wt% of MNPs incorporation. The PGS nanocomposites with 0, 1 and 10 wt% MNPs were referred to as 0%, 1% and 10%, respectively. High-power sonicator combined with vortex was used to disperse MNPs in PGS solution. The optimized amplitude, energy and time frame for dispersing MNPs with high-power sonicator was 80%, 22 watts and 20 minutes with 5 seconds on and 5 seconds off, respectively, under the protection of ice to prevent from heat-up. PGS-MNPs suspension was then dropped into the PFA mold to cover the salt template homogeneously. After air dried for 1 hour, the PFA mold was placed into the vacuum oven and kept at 10 torr absolute pressure and room temperature for 1 h to remove all the ethanol residues. The temperature of the vacuum oven was then increased to 120 °C to cure PGS nanocomposite for 48 hours. PGS nanocomposite together with the salt template was taken out from the PFA mold and NaCl was dissolved in deionized water (DI water) with water change twice in 24 hours. PGS nanocomposite was then air-dried in room temperature for 12 hours.

Characterization of Porous Magnetic PGS Nanocomposites

Porous PGS nanocomposites were characterized with scanning electron microscope (SEM, Nova NanoSEM 450, FEI, Hillsboro, OR) with spot size 3 and acceleration voltage of 2 kV. The magnification of $50\times$ was used to detect the surface features and cross section thickness of porous PGS nanocomposites.

8.2.2 COMSOL Simulation of Different Coil Designs

COMSOL simulation was conducted to determine the type of coil design for this study as described in previous chapter. To achieve an EMF intensity of 1 mT in our experiment, a current of 500 mA was used. The diameter of the coil was set to 14.88 cm, which provides a sizeable area of uniform EMF intensity to encompass the entire well plate, while still minimizing the size of the overall coil system. A default conductivity of 6.00×10^7 S/m was used for the copper wire at approximately room temperature. Also, using the coil diameter chosen above, the coil positions were determined from the coil spacing from center of system relations for each coil design.

8.2.3 Fabrication and Testing of EMF Generating Device and Shielding Box

The EMF generating device and shielding box was fabricated and tested as described in the previous chapter. Briefly, a programmable DC power supply and a gaussmeter (Lakeshore) was used for the test. The EMF generating device was placed into the shielding box and connected with the DC power supply that drives the current flow through the coil

system via lead cords plugged into metal sockets attached near the base of the coil frames. The ends of the copper wires wound around the coil frames are bent into hooks that make contact with the metal sockets, establishing the closed circuitry necessary for current flow. With the current set to 500 mA and 1 A, the EMF intensities inside the coils in the shielding box and outside the shielding box were all tested with the gaussmeter. The tested EMF intensities were compared with COMSOL simulation results, and the EMF generating device was tested to have no heating effects.

8.2.4 Cell Culture in the EMF Generating Device and Shielding Box

Pre-treatment of Porous Magnetic PGS Nanocomposites

In our previous study, we were able to prove that the presence of PGS residuals caused cell death and morphology change of BMSCs, so a pre-treatment of pure PGS and PGS nanocomposites was critical for cell study. Hence, pre-treatment of porous magnetic PGS nanocomposite was conducted to remove the reaction residuals before cell study. Before the pre-treatment, each type of nanocomposite was cut into 7×7 mm scaffolds triplicate samples were randomly selected for cell culture. The process of pre-treatment was modified from Wang et al. [146]. Briefly, all the scaffolds were soaked serially in 70, 50 and 25% ethanol for 1 hour each in a laminar flow hood, followed by soaking twice in PBS for 1 hour each, and conditioned in DMEM media supplemented with 10% FBS and 1% Penicillin/Streptomycin (complete DMEM) in standard cell culture environment (i.e., 37 °C, 5%

CO₂/95% air, humidified environment) for 24 hours before cell study. Media pH, scaffold dry mass and surface morphology were characterized after the pre-treatment process.

Culture of BMSCs

Bone marrow-derived mesenchymal stem cells (BMSCs) were cultured in T75 flask (Fisher Scientific) until confluent. EMF generating device and shielding box were sprayed and wiped with 70% ethanol, respectively, and placed in laminar flow hood afterwards for overnight UV-sterilization. The device and shielding box were then placed in cell culture incubator 24 hours ahead of cell seeding in order to reach temperature stabilization.

Confluent flask of BMSCs was washed with PBS to remove dead cells and detached with trypsin-EDTA (#25200-056, Life Technologies, Carlsbad, CA). Fresh media was added to the vial to make cell suspension and the cells were count manually with a hemocytometer (#1475, Reichert Bright-Line, Hausser Scientific, Horsham, PA) under microscope. Cell seeding density was set to 10,000 cells/cm² based on previous publications [166, 167], in order to reach confluency after 48 hours. Cells were seeded on magnetic PGS nanocomposite scaffolds and control wells in 12-well chamber slides (ibidi) in triplicate groups and cultured in osteogenic media (complete DMEM supplemented with 100 nM Dexamethasone, 10 mM beta-glycerophosphate and 50 µg/mL L-ascorbic acid) in standard condition (i.e., 37 °C, 5% CO₂/95% air, humidified environment) without EMF for 48 hours to get initial density. After 48 hours, the EMF device was connected with external power supply that could provide 30 Hz and 500 mA triangle wave. Half of the groups were placed on top of the

platform inside EMF generating device for 21-day culture, and another half without EMF for 21-day culture in comparison. The control groups were placed in the same incubator under the EMF shielding box with a distance of 25 cm, which position was tested to be not affected by the EMF generating device.

Cell Proliferation Characterization

BMSCs were fixed and stained at 48 hours prior to the application of external EMF, and 1 week, 3 weeks after the application of external EMF to examine their proliferation and morphology. Adherent BMSCs were fixed with 4% paraformaldehyde (PFA; #15714-S, Electron Microscopy Science, Hatfield, PA) solution, stained with Alexa Fluor 488 solution (#A12379, Life Technologies, Carlsbad, CA) for F-actin visualization and Hoechst 33342 solution (#H3570, Life Technologies, Carlsbad, CA) for nucleus visualization. The cells were then imaged using an inverted fluorescence microscope (Nikon Ti-S eclipse, Nikon, Japan).

Calcium Deposition and Iron Concentration Test

Calcium deposition and iron content was also tested with inductively coupled plasma optical emission spectrometry (ICP-OES, Model OptimaTM 8300, PerkinElmer Inc., Waltham, MA). 2% nitric acid was used to dissolve mineral deposits on the samples and on the slide and then the samples were diluted 100 times for calcium ion concentration and iron ion concentration test with ICP-OES. The test results were compared with colorimetric test results.

Cell differentiation colorimetric characterization

BMSCs were lysed with freeze-thaw method. At week 1 and 3, cell culture media was removed and wells including cells and scaffolds were rinsed with PBS and DI water each for one time. Same volume of DI water was added to each group, repeatedly froze in -20 °C freezer for 3 hours and then thaw at room temperature for 4 cycles. The cell lysates were then examined with quantification methods of calcium deposition, ALP activity, protein content and collagen secretion.

QuantiChromTM Calcium Assay Kit (#DICA-500, BioAssay Systems) was used for the quantification of calcium ions in the study. Each reagent was equilibrated to room temperature prior to use. 5 μ L diluted standards and cell lysates were transferred into wells of a clear-bottom 96 well plate, and 200 μ L working reagent was added to each well and tap lightly to mix. The mixture was incubated at room temperature for 3 minutes and the optical density was read at 612 nm using the micro-plate reader. Calcium ion concentration was calculated from the subtraction of blank controls.

QuantiChromTM Alkaline Phosphatase Assay Kit (#DALP-250, BioAssay Systems) was used for the quantification of ALP activities in the study. Each reagent was equilibrated to room temperature prior to use. 200 μ L DI water and calibrator was pipetted into separate wells of a 96-well plate as control groups. 50 μ L cell lysates from each group per time point were pipetted into 96-well plate and 150 μ L working solution was added to each well to mix with samples. The mixture was tapped briefly and absorbance

was read at $t = 0$ and $t = 4$ minutes at wavelength of 405 nm. ALP activity was calculated with the equation provided by the company.

Total protein content was quantified with a Micro BCA Protein Assay Kit (#23235, Thermo Fisher Scientific). 150 μL of series standard protein solution and unknown cell lysates were pipetted into 96-well plates in triplicate, and 150 μL of working reagent was added to each well and the plate was mixed by shaking for 30 seconds. The plate was covered using a sealing tape and incubated at 37 °C for 2 hours. After cooled to room temperature, the absorbance of all the samples was read at 562 nm with a micro-plate reader. A standard curve was plotted based on standard protein concentrations and the total protein concentration of each sample was calculated with the standard curve and presented in $\mu\text{g/mL}$.

Sirius Red staining dye was also used for the quantification of collagen secretion. Standard collagen solution was prepared by dissolving lyophilized bovine type I collagen (#1200-02, SouthernBiotech) into DI water and serially diluted into different concentrations. 50 μL of standard collagen solution and cell lysates were pipetted into a 96-well plate and dried in an incu-shaker (Incu-shaker Mini) for 2 days at 37 °C and 100 rpm. Then 50 μL of Sirius Red staining dye was added to each well and incubated at room temperature for 1 hour. After the dye was removed, all wells were rinsed with 0.01 M HCl for five times to remove unbound stain and the stain was collected by washing with 0.1 M NaOH for 5 minutes. The collected stain was then transferred to another 96-well plate and the

absorbance was read in the micro-plate reader at 540 nm. A standard curve was plotted and collagen content of the samples was calculated and expressed as mg/cm².

Elemental Composite Test Post Culture

Magnetic PGS nanocomposites post culture was rinsed with PBS and DI water each for one time, and air-dried at room temperature. The samples were sputter coated for conductivity and examined with SEM and EDS detector with spot size 5 and acceleration voltage of 15 kV. The magnification of 50× was used to detect the majority area of samples for representative analysis results. Element distribution was mapped with EDS analysis.

8.2.5 Statistical Analysis

Experiments data were analyzed in R using one-way analysis of variance (ANOVA) followed by the Tukey highest significant difference post hoc test. Statistical significant difference was considered at $p < 0.05$.

8.3 Results

8.3.1 Characterization of Porous Magnetic PGS Nanocomposites

SEM analysis showed the presence of interconnected pores with main pore size corresponding to the NaCl salt size which ranged from 125-150 μm . The pore distribution was consistent on the surface for all groups (Figure 8.1a). The thickness of each group of scaffold analyzed with SEM showed that 0% nanocomposite showed lower thickness which

was $293 \pm 16 \mu\text{m}$, while 1% and 10% were thicker with the thickness of $329 \pm 28 \mu\text{m}$ and $329 \pm 40 \mu\text{m}$, respectively (Figure 8.1b).

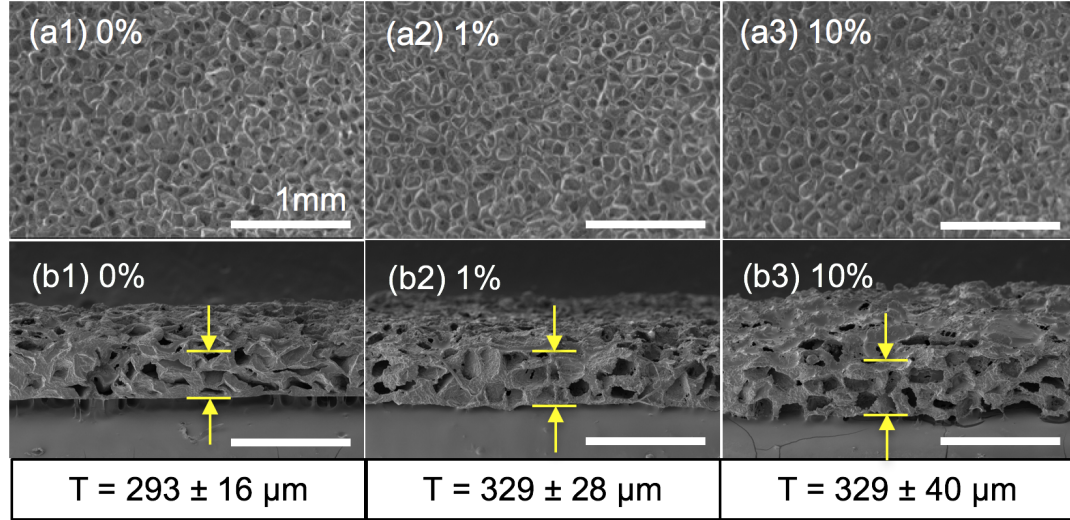


Figure 8.1: SEM characterization of 0%, 1% and 10% magnetic PGS nanocomposites. (a) SEM micrographs showing the porous structure of magnetic PGS nanocomposites on the surface. Original magnification = $50\times$, scale bar = 1 mm for all images. (b) SEM micrographs showing the porous structure of the cross section and thickness of corresponding magnetic PGS nanocomposites. The yellow arrows indicated the cross section of the nanocomposites. Original magnification = $50\times$, scale bar = 1 mm.

8.3.2 COMSOL Simulation of Different Coil Designs

Results showed that while both the Lee-Whiting 4 circular coil system and Merritt 4 square coil system were able to achieve the same highest field intensity in the center of the system, the Lee-Whiting design required less spacing between the coils and fewer turns per coil, allowing for a more efficient use of space and materials. In conclusion, the Lee-Whiting 4 circular coil system can provide the desired EMF intensity, uniform area and coil spacing for the design of EMF generating device, which ensured the uniform exposure of sufficient

amount of chamber slides to the EMF generated by the device while the device can still fit in the incubator for the future test of cell functions.

8.3.3 Testing of EMF Generating Device and Shielding Box

From the gaussmeter testing results, the real EMF intensity generated at the center of the device was 11.1 ± 0.7 gauss (1.11 ± 0.07 mT) when the current was 500 mA and 20.2 ± 0.6 gauss (2.02 ± 0.06 mT) when the current was 1 A, which were consistent with the COMSOL simulation results of 10 gauss (1 mT) at 500 mA and 20 gauss (2 mT) at 1 A, respectively. The table of the simulation comparison also showed that EMF generated by single 80-turn coil and single 180-turn coil were consistently match with the COMSOL simulation results.

When apply the shielding box, the field intensity at the center of the device was not affected while the field intensity outside the shielding box was reduced significantly. When the current was set to 1 A, the EMF intensity at 10 cm away from the outside of the three-layered shielding box was 0.4 gauss, which was 0.04 mT and in the range of geomagnetic field. The test results showed that the EMF generating device could provide the field intensity as needed and consistent with the COMSOL simulation results, and the shielding box could shielding the inside field from affecting the outside environment.

8.3.4 Cell Proliferationi Characterization

Cells reached confluency after 48 hours of culture prior to EMF exposure. Cell density and morphology was similar between each groups after 1 week of culture with and

without EMF exposure (Figure 8.2). Cell morphology on the scaffolds was consistent with our previous studies. Cells appear to be 3D lay-out and penetrated into the porous area of the scaffold. And cells in the cell only group and MNPs groups were normal irregular morphology and 2D spread out on the bottom of each well. Cell density in all the nanocomposite groups were similar to cell only control group, while the cell density in 0.1 mg and 1.2 mg MNPs groups were lower than cell only control, however, there was no significant difference (Figure 4).

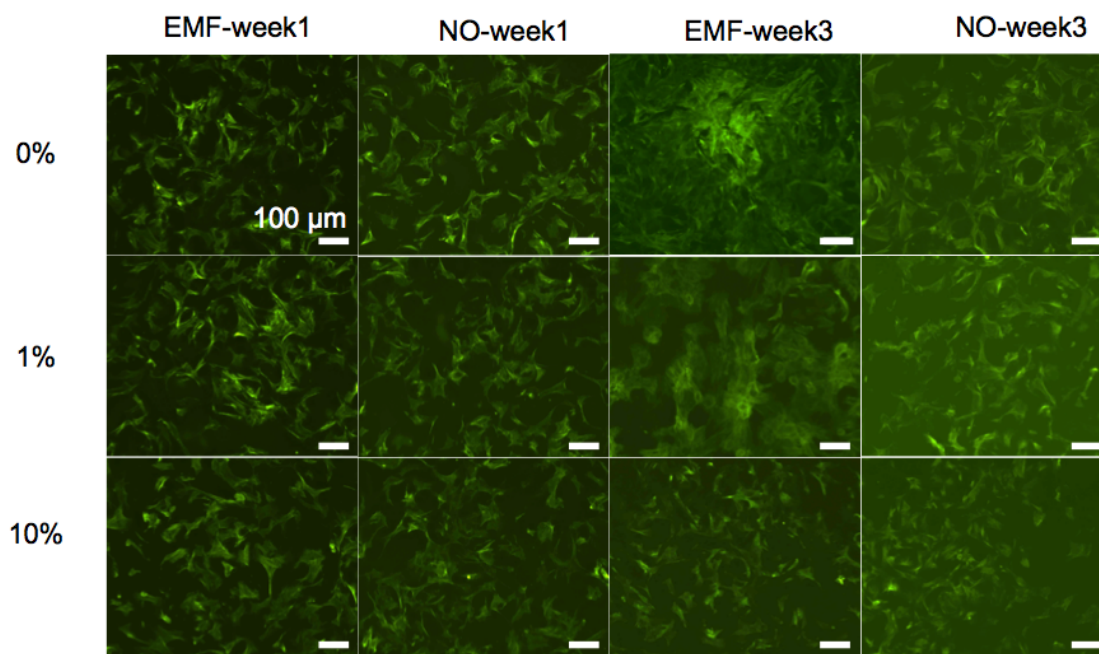


Figure 8.2: Fluorescence images of BMSC after 1 week and 3 weeks of culture in direct contact with magnetic PGS nanocomposites. Scale bar = 50 μm for 0%, 1%, 10% and cell groups. Scale bar = 100 μm for MNPs groups.

At week 3, cell aggregates were observed in all groups (Figure 8.3). Those aggregates were 3D-shpere-shaped clumps, and the aggregation of cells were pockets of layer-by-

layer cell nodules with cells sheets around them. The center of the aggregates was too thick to be imaged clearly with a single focus. There were less cell aggregates observed on the bottom of the wells compared to on the scaffolds. The individual cells in the cell only group were similar to those observed at week 1, however, the cells on the bottom of the wells in the nanocomposite groups had fluffy-shaped cytoskeleton, and protein interconnection nodules were observed in those groups, indicating different cell behaviors. BMSCs in MNPs groups presented network-like cells and all the cells had much elongated pseudopodia that appeared to be neuron look. At weeks 3, only the cell only control group had significantly increased cell density. Cell density in the 0% and 1% groups exposed to EMF were higher than week 1 time point but 10% was lower. Cell density in the 0%, 1% 10% and MNPs groups without exposure to EMF had similar cell density compared with 1 week time point. Cell density in cell only group with the exposure to EMF at week 3 time point was significantly higher than the rest of the groups at the same time point (Figure 8.4).

8.3.5 ICP-OES Test Results of Calcium and Iron Ion Concentration

Calcium ion concentration in the 0%, 1% and 10% groups were higher than cell only and MNPs groups at the same time point and 10% groups at week 3 with and without exposure to EMF was significantly higher than cell only and MNPs groups (Figure 8.5a). There was a trend of calcium ion concentration increase from 0% group to 10% group, but there was no significant difference. The result showed a higher mineral deposition in

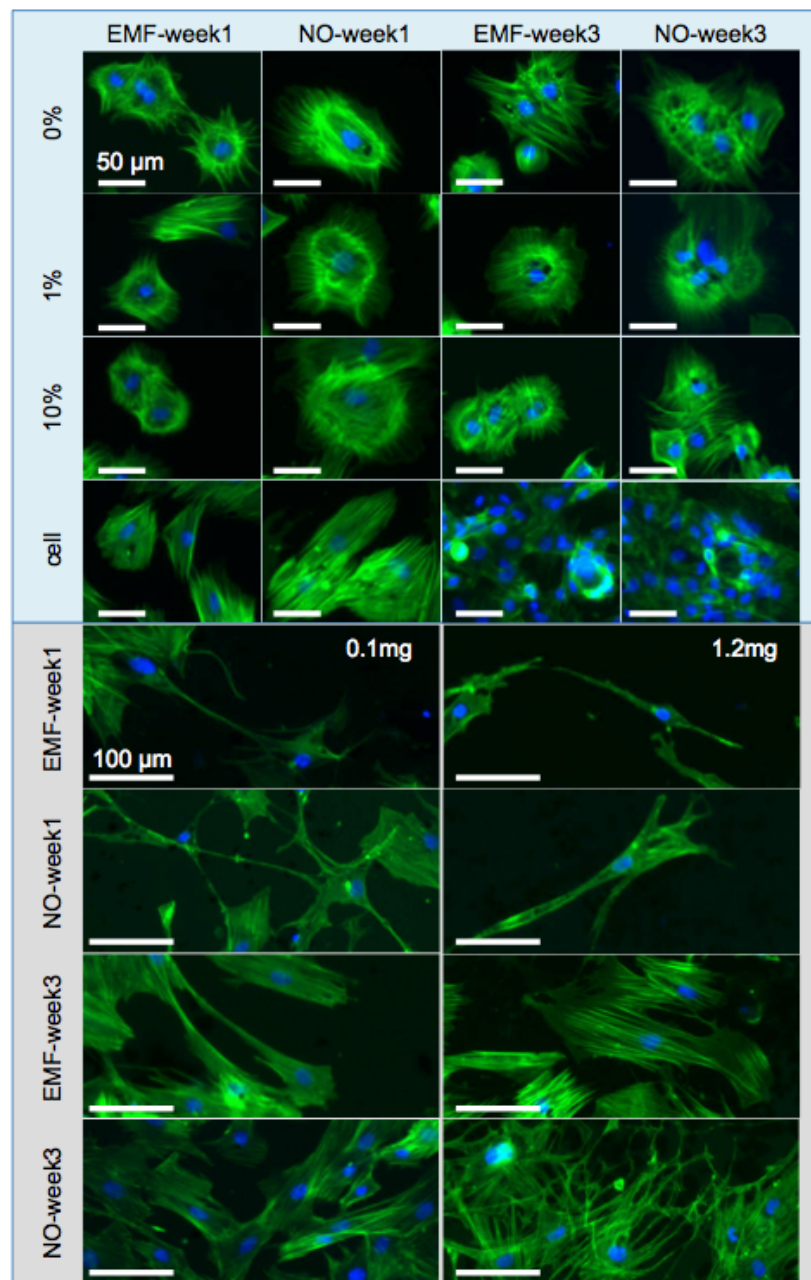


Figure 8.3: Fluorescence images of BMSC after 1 week and 3 weeks of culture around magnetic PGS nanocomposites (indirect contact) and on the slides. Scale bar = 100 μm .

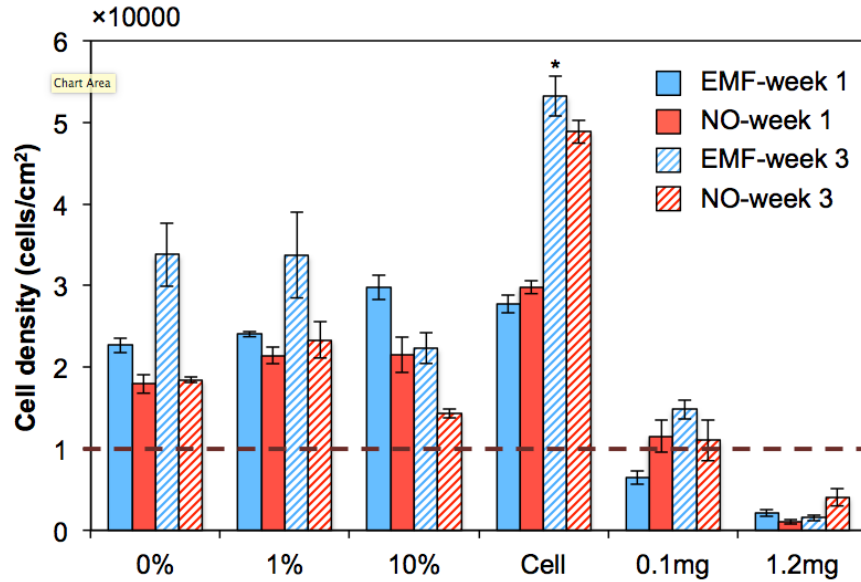


Figure 8.4: The overall density of BMSC in each group after 1 week and 3 weeks of culture with and without magnetic PGS nanocomposites and EMF. Values are means $\pm$ standard error of the means, $n = 3$. * $p < 0.05$ compared to all the rest of the groups at the same time point.

the nanocomposite groups compared to cell only group and MNPs group. There was no significant difference between the EMF group and no EMF group at the same time point in each study group.

Iron ion concentration was low in 0% group and cell group with and without exposure to EMF at week 1 and week 3 time point (Figure 8.6). Iron ion concentration in 1% group and 0.1 mg group were slightly higher than 0% and cell only group, but there were no obvious changes along with time. 10% group had significantly higher iron ion concentration compared to 0%, 1%, cell only and 0.1 mg groups at all time points with and without EMF exposure. 1.2 mg group at week 1 without exposure to EMF had significantly higher iron ion concentration compared to all the rest of the groups at the same

time point, and 1.2 mg group at week 3 with exposure to EMF had significantly higher iron ion concentration compared to all the rest of the groups.

8.3.6 Cell Differentiation Colorimetric Characterization

Calcium ion concentration was similar in all groups at week 1, and at week 3, there was a significant increase in cell only, 0.1 mg and 1.2 mg groups compared to the nanocomposite groups at the same time point (Figure 8.5b). The calcium ion concentration in the nanocomposite groups had no obvious change along with time, indicating a positive and continuous consumption of calcium from media, while the significant increase of calcium ion concentration in cell only and MNPs groups indicated active intake and accumulation of calcium from media but without further mineral deposition at the same pace. Overall calcium concentration in the 10% group at week 3 with and without EMF exposure was significantly higher than the cell only and MNPs groups (Figure 8.5c). The overall calcium concentration in the nanocomposite groups remained similar and cell only and MNPs groups remained similar at each time point with and without exposure to EMF.

Total protein concentration in the nanocomposite groups were higher than cell only and MNPs groups at each time point with and without exposure to EMF and the cell only and MNPs had similar protein concentration at conditions at each time point (Figure 8.7). Protein concentration in the 0% group remained similar at each time point while 1% and 10% groups had an overall trend of slightly increase of protein concentration. The

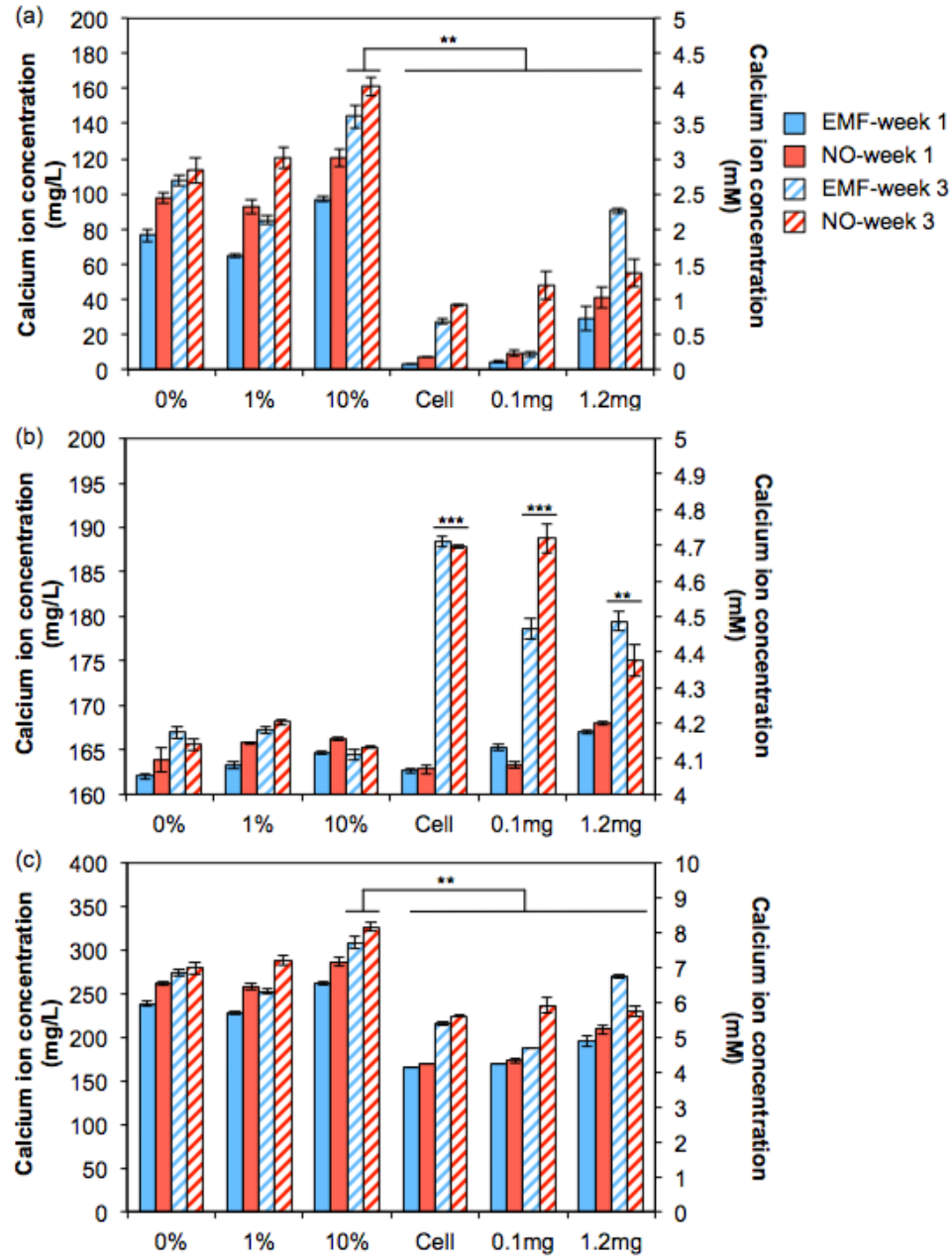


Figure 8.5: Calcium ion concentration quantification in each group after 1 week and 3 weeks of culture with BMSCs. (a) ICP-OES analysis results of calcium ion concentration in the mineral deposits in each group after 1 week and 3 weeks of culture. (b) Calcium ion concentration in cell lysates in each group analyzed by calcium detection kit after 1 week and 3 weeks of culture. (c) Overall calcium ion concentration in each group analyzed by ICP-OES and calcium detection kit. Values are means $\pm$ standard error of the means, $n = 3$. ** $p < 0.01$, *** $p < 0.001$ compared to all the rest of the groups.

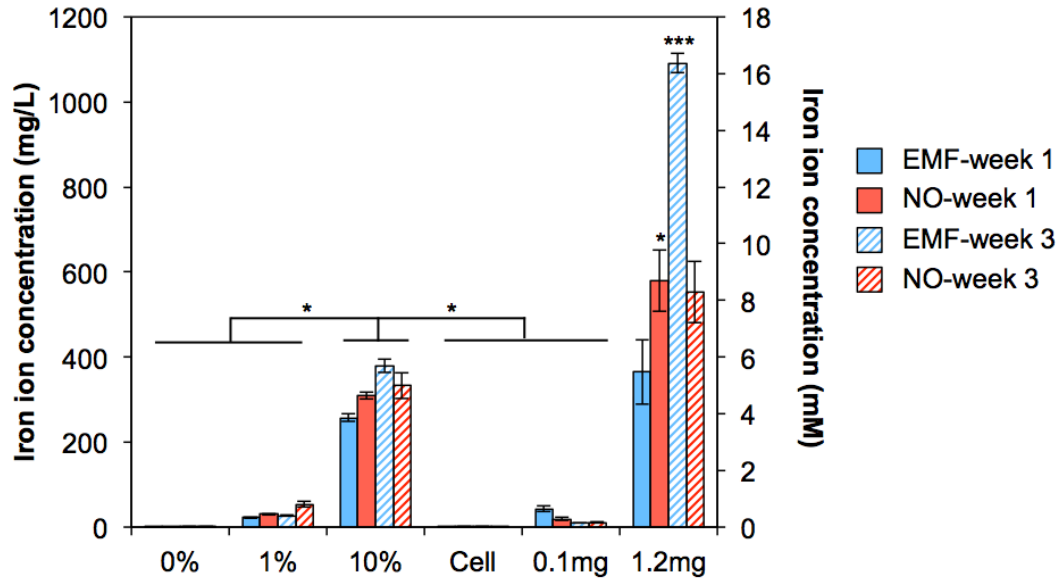


Figure 8.6: ICP-OES analysis results of iron ion concentration in the mineral deposits in each group after 1 week and 3 weeks of culture. Values are means $\pm$ standard error of the means, $n = 3$. * $p < 0.05$ compared to all the rest of the groups at the same time point, *** $p < 0.001$ compared to all the rest of the groups

protein concentrations in 1% and 10% groups at week 3 with and without EMF exposure were all significantly higher than the rest of the groups at the same time point.

From the collagen concentration diagram, collagen secretion in the nanocomposite groups was significantly higher than the cell only and MNPs groups at each time point with and without exposure to EMF except the 0.1 mg group (Figure 8.8). There was no difference in collagen concentration among the nanocomposite groups and no difference between the cell only and MNPs groups. There was no significant increase in collagen concentration along with time in all groups.

ALP activities were low in all groups but increase was shown in the 0% and 1% groups. The 10%, cell only and MNPs groups all had similar ALP activities at week 1 and

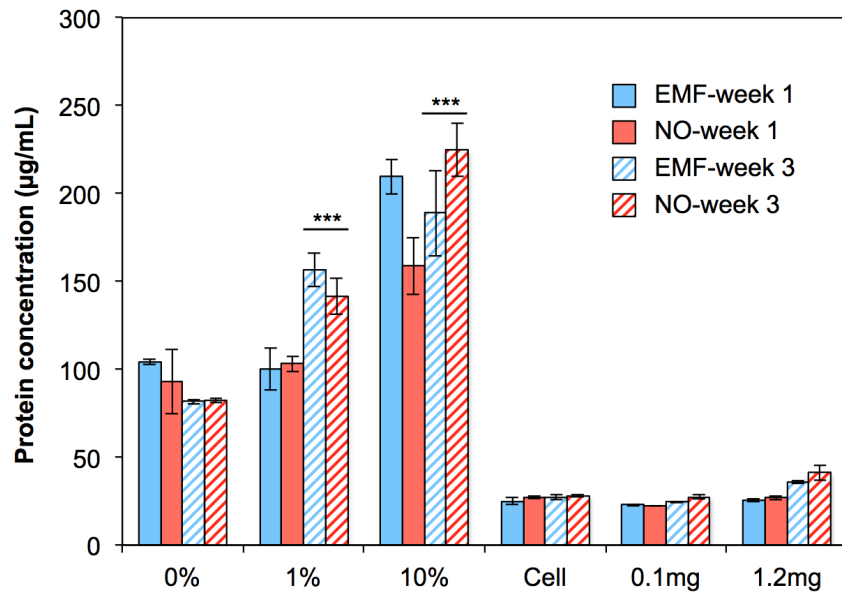


Figure 8.7: Total protein concentration in cell lysates in each group after 1 week and 3 weeks of culture quantified with Micro BCA kit. Values are means $\pm$ standard error of the means, n = 3. ***p<0.001 compared to all the rest of the groups at the same time point.

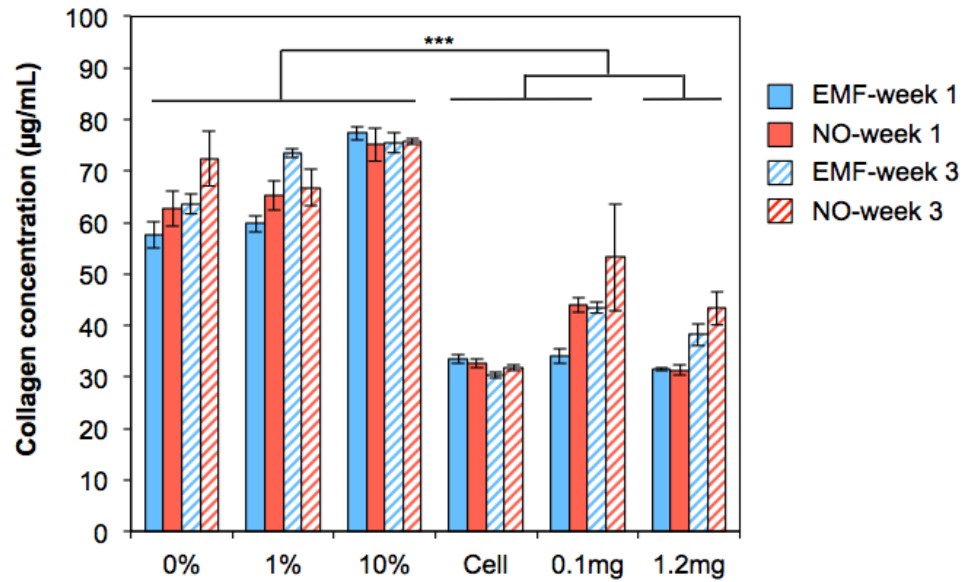


Figure 8.8: Total collagen concentration in cell lysates in each group after 1 week and 3 weeks of culture quantified with Sirius Red staining assay. Values are means $\pm$ standard error of the means, n = 3. ***p<0.001.

week 3 (Figure 8.9). The ALP activity in 0% group at week 3 with exposure to EMF was significantly higher compared all the rest of the groups at the same time point.

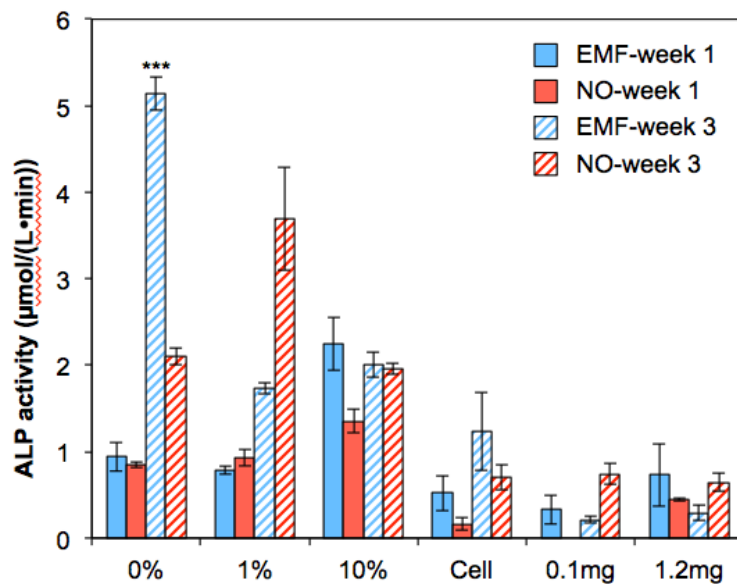


Figure 8.9: ALP activities of each group of BMSCs after 1 week and 3 weeks of culture quantified with Micro BCA kit. Values are means $\pm$ standard error of the means, $n = 3$. *** $p < 0.001$ compared to all the rest of the groups at the same time point.

8.3.7 Scaffold Surface Elemental Composition Characterization

The 0%, 1% and 10% nanocomposite post 1 week and 3 weeks of culture with BMSCs all had similar type of elemental composition but different atomic percentages (at.%) (Figure 8.10a). The major elements include carbon (C), oxygen (O), calcium (Ca), iron (Fe), phosphorus (P), sulfate (S) and sodium (Na). The percentage of C was in the range of 70 - 80 at.% and O was in the range of 20 - 25 at.% in all groups. A magnified diagram showing the percentage of Ca, Fe and P was presented in Figure 8.10b. 0% groups were proved to have no iron content and there was almost no calcium deposition detected at

week1 but about 1 at.% at week 3. Fe percentage was higher in the 10% groups compared to 1% groups. Calcium percentage increased at week 3 compared to week 1 and 1% groups at week 3 with and without exposure to EMF had higher calcium content at 1.7 at.% and 2.5 at.% compared to all the rest of the groups (Figure 8.11). The ratio of P percentage to Ca percentage was always 1:3.

8.4 Discussion

8.4.1 BMSC Proliferation Behavior

Fluorescence images in Figure 8.2 showed that cell morphology did not change within the first week in each group, however, there were cell aggregates formed in each group at week 3 time point, indicating a positive formation of bone nodules. There were less cell aggregates visualized on the porous scaffold compared to the cell only groups, which might due to the porous structure of the nanocomposites that might allow cell migration through the pores, and the nanocomposites had lower surface toughness compared to glass chamber slides that might affect cell behavior as well. Thus, assays to quantify cell proliferation and differentiation behavior is necessary. Besides, cells density in the MNPs groups were much lower than the nanocomposite and cell only groups, and the cells showed neuron-like morphology indicated a possibility of MNPs influence in cell functions. Cells on the nanocomposites were 3D shaped to adapt to the topography of the nanocomposites, but

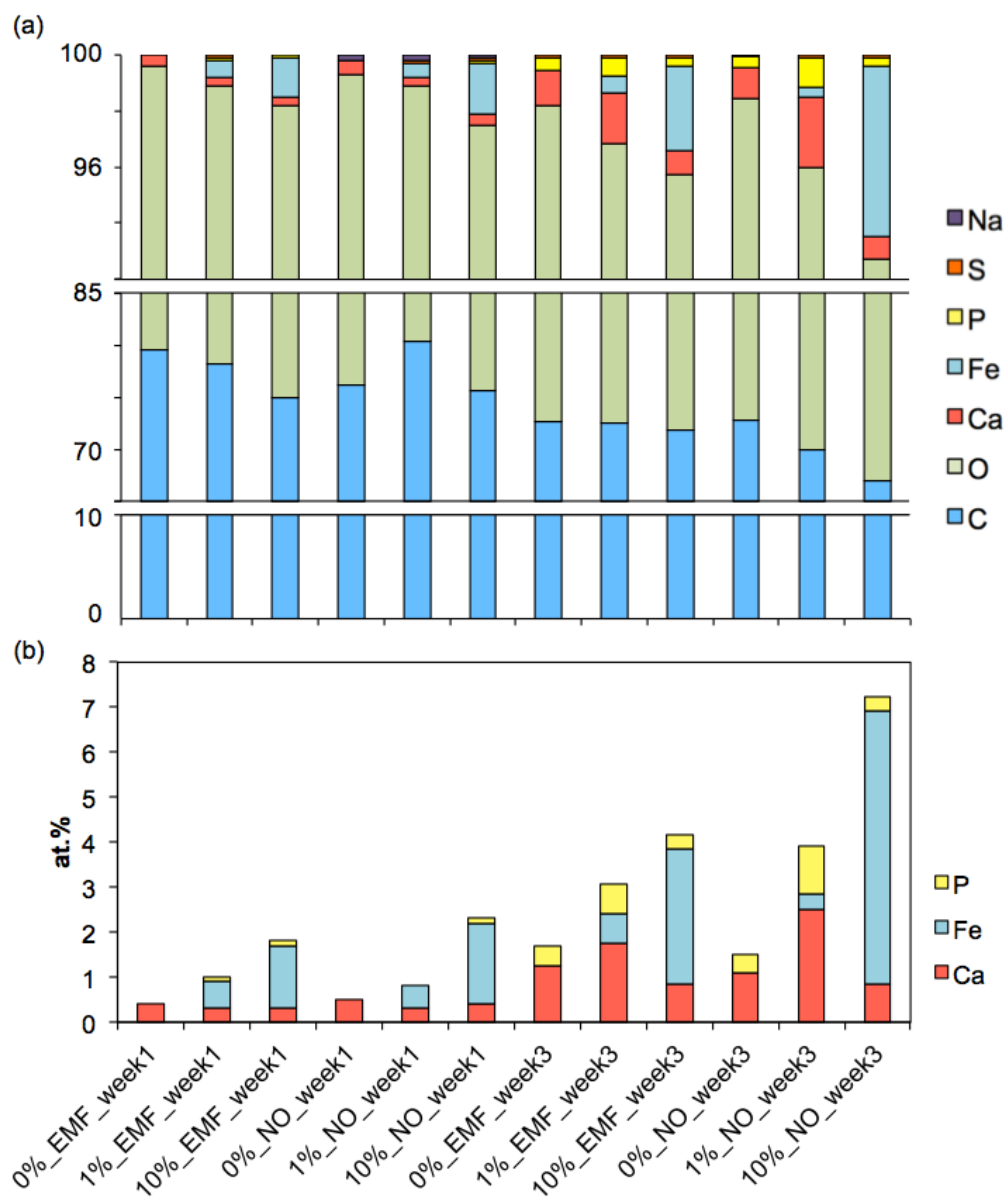


Figure 8.10: (a) Surface elemental composition (at.%) quantified through EDS area analysis on SEM images at original magnification = $50\times$. (b) Enlarged EDS analysis results of P, Fe and Ca shown in (a).

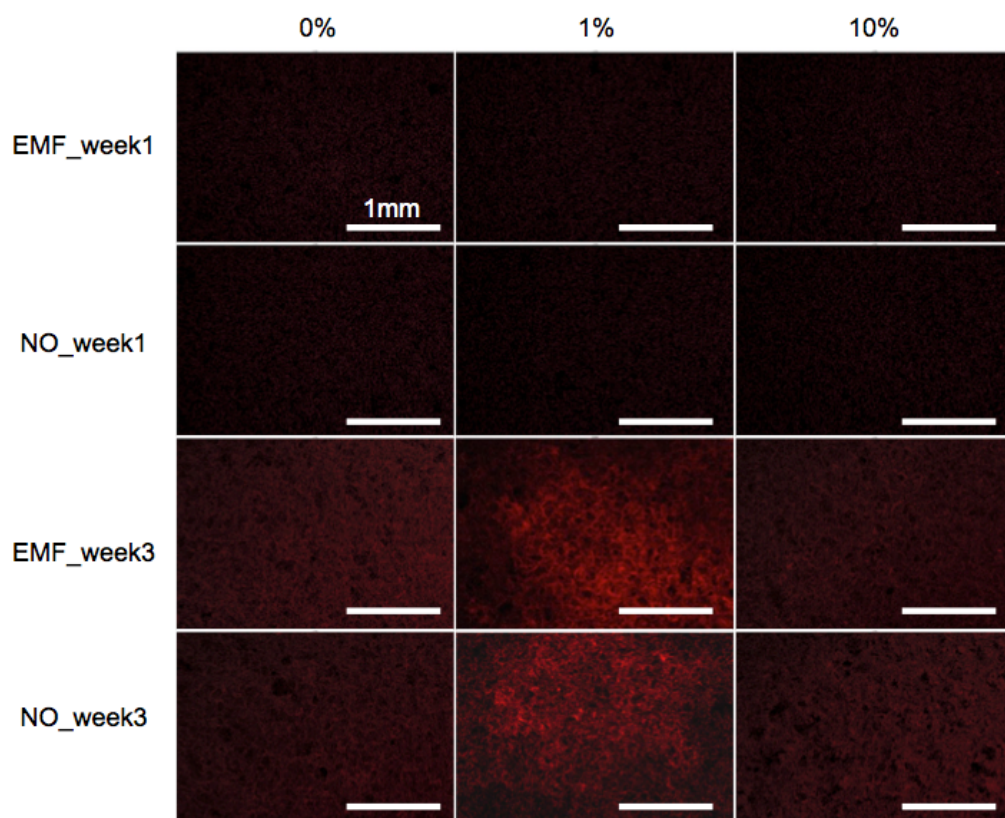


Figure 8.11: EDS elemental distribution map of Ca $K\alpha$ lines of magnetic PGS nanocomposites after 1 week and 3 weeks of direct culture with BMSC in osteogenic media. Original magnification = $50\times$, scale bar = 1 mm for all images.

the cells on the bottom of the same well showed different morphology compared to cell only groups were of interest, which might indicated a different type of cell function.

8.4.2 Cell Function Behaviors

Calcium ion concentration detected by ICP-OES showed that the 10% group at week 3 with and without EMF exposure had highest amount of calcium deposition and the cell only and MNPs had the lowest calcium deposition. The colorimetric assay detected by calcium quantification kit showed highest calcium concentration in the cell only and MNPs groups, indicating active calcium intake and accumulation inside the cells without calcium deposition at the same pace. The overall calcium concentration showed significant higher value in the 10% group with and without exposure to EMF and higher value in all nanocomposite groups compared to cell only and MNPs groups. The results demonstrated active calcium accumulation in BMSCs in all study groups, however, the calcium consumption and deposition rate was much higher in the nanocomposite groups compared to the cell only and MNPs groups. ALP activity results confirmed that the BMSCs in the nanocomposite groups had higher ALP activity than cell only and MNPs groups, which is consistent with the calcium concentration results.

Protein concentration and collagen concentration were all higher in the nanocomposite groups compared to cell only and MNPs groups, which is consistent with ALP activity results as well. The higher thickness of 10% nanocomposite compared to 0% and 1% nanocomposite might contributed to protein and collagen retention. Considering the

higher cell density in the cell only group, the lower calcium deposition, protein and collagen secretion might result from a low cell activity compared to the nanocomposite groups. Besides, our previous study showed that the surface-erosion mechanism might be one of the reasons of the lower cell density on the nanocomposite compared to cell only group since the initial attached cells could be peeled-off together with the degradation of the PGS nanocomposites. As for the MNPs groups, the cell density was low compared to all the rest of the groups, thus the low activity in ALP, calcium deposition, protein and collagen secretion was expected. The magnetic PGS nanocomposite scaffolds increased individual cell activity. However, this total protein assay detects all proteins but not able to differentiate various type of proteins, which need further confirmation.

Calcium accumulation on the 1% nanocomposites shown in Figure 10 might indicate a better local calcium deposition environment compared to the 0% and 10% nanocomposites, however, this advantage is not significant enough to help increase or speed up calcium deposition. Iron concentration was higher in the 10% group and 1.2 mg group, indicating iron ion and MNPs release from the nanocomposite along with time. The iron release from the nanocomposites did not affect the overall behavior of the BMSCs, however, the higher content of iron released from the MNPs group might be one of the reasons that affect BMSC functions.

Through all the study results, BMSC behaved better on the 1% and 10% nanocomposites compared to the 0% group, and in considering of the benefit of external magnetic manipulation, the 10% group that had the best supporting function among all the groups

and able to respond to magnetic field is a potential candidate for future bone and cartilage tissue engineering applications.

8.5 Conclusions

The magnetic PGS nanocomposites supported BMSC proliferation and differentiation behavior in the three-week study. BMSC had higher calcium deposition, protein and collagen secretion and ALP activity compared to cell only and MNPs groups although with a lower cell density than cell only group. The results indicated increased cell activity in the nanocomposite groups compared to cell only group. The three weeks exposure of EMF did not affect BMSC functions compared to the groups without exposure to EMF. All in all, the magnetic PGS nanocomposites could be a potential candidate in future bone and cartilage tissue engineering applications and able to be remote manipulated by external magnetic field without side effects to BMSC functions.

8.6 Acknowledgements

We would like to thank Dr. Nissim Amos for his kind help with the use of gaussmeter. We thank Junchao Wang and Dr. William Grover for their kind help on the final coding of Arduino Uno R3. We thank Central Facility for Advanced Microscopy (CFAMM) at the University of California, Riverside, for the use of SEM and Ilkeun Lee and Dr. Krassimir Bozhilov for the training on SEM.

Chapter 9

Conclusions

This project presented here focused on defining the ability of magnetic nanocomposite scaffolds coupled with magnetic fields to induce BMSCs differentiation for bone and cartilage tissue regeneration applications.

The study of PVA-coated MNPs showed that all groups of MNPs exhibited sphere shape with diameter of 5-10 nm, similar crystallinity and superparamagnetism. The size, shape, crystallinity and magnetism of MNPs were not affected by the weight percent of PVA coating, indicating the preservation of particle properties. However, with higher weight percent of PVA coating, less aggregates of MNPs were formed and better dispersion in polymer solution were achieved. Thus, 30% PVA-coated magnetic nanoparticles were chosen for the synthesis of magnetic nanocomposites for homogeneous dispersion in polymer substrates.

MagGel was synthesized with the incorporation of 30% PVA-coated MNPs for potential cartilage tissue engineering applications. Because the MagGel responded to an external magnetic field, it could be used to direct the scaffold to the exact cartilage defect

site remotely using an external magnet. Moreover, the MagGel showed similar microstructure and chemistry as hyaline cartilage and was cytocompatible with BMSCs *in vitro*, as demonstrated by the increased BMSC viability when compared with the Gel in the 24 h culture. However, the MagGel degraded within 24 hours of culture with cells, thus a magnetic nanocomposite that not only has good cytocompatibility but also longer degradation time-frame is desired for *in vivo* applications as supporting scaffold for tissue healing.

PGS was brought up as alternative candidates for potential tissue engineering applications. Unreacted monomers resided in the magnetic PGS nanocomposites after curing resulted in a relatively acidic environment during direct contact with cells and caused cell death. A series soaking pretreatment process helped remove the unreacted monomers in magnetic PGS nanocomposites before direct contact with BMSCs. However, the smooth surface and surface degradation mechanism of 2D magnetic PGS nanocomposites are not ideal for cell attachment. An investigation on the porous magnetic PGS nanocomposites was conducted to increase cell adhesion. The magnetic response speed of the nanocomposites with different ratio of MNPs incorporation is beneficial for the determination of MNPs percentage in future clinical magnetic implants.

Porous structure helped increase BMSC attachment on PGS nanocomposites and BMSCs favors the porous area. BMSCs grew on different group of nanocomposites did not show significant differences in their morphology and density and BMSCs were able to migrate inside the scaffold and survive for 72 hours. However, surface degradation of the nanocomposites resulted in the low increase of BMSC density grew on each group of

nanocomposite after 72 hours when compared to the 24 hours of culture. Experiments showed that small amount of MNPs incorporation such as 0.5 wt% would increase the degradation rate of PGS while a ratio higher than that would help decrease the degradation rate. MNPs can be used to adjust the degradation rate of PGS so as to be compatible with the new tissue growth rate of the defect tissue site. The preserved magnetic responsive ability of the nanocomposites after culture made it possible to react to external magnetic field stimuli even after implantation for several days and this would help the study on the synergistic effects of external magnetic fields and MNPs on stem cell proliferation and differentiation.

The influence of magnetic fields on BMSC functions was tested, including two moderate static magnetic fields and one moderate electromagnetic field. The 72-hour culture results showed that the SMFs with a high intensity of 340 mT increased the average cell proliferation as compared with the field with a low intensity of 14 mT, but there was no significant difference detected. The statistical analysis of the 72-hour culture results showed that the SMFs with a high intensity of 340 mT and low intensity of 14 mT had no influence on cell morphologies. Cells in high and low magnetic field intensity wells displayed a variety of morphologies and their aspect ratios were similar, which indicated the cell morphology did not change with respect to magnetic field intensity.

The study of BMSC behaviors when exposed to EMF showed that EMF helped increase calcium deposition in osteogenic media groups while there was no significant difference of protein and collagen secretion and ALP activities between EMF and no EMF

groups cultured with osteogenic media or base media. However, the supplementation with Dexamethasone, beta-glycerophosphate and L-ascorbic acid helped increase all cell differentiation activities including calcium deposition, protein and collagen secretion and ALP activities.

The three week study of EMF influence on the differentiation behaviors of BMSC cultured on magnetic PGS nanocomposites showed that BMSC had higher calcium deposition, protein and collagen secretion and ALP activity compared to cell only and MNPs groups. The results indicated increased cell activity in the nanocomposite groups compared to cell only group and the PGS nanocomposites were able to support cell proliferation and differentiation for 3 weeks without losing structural integrity. The three weeks exposure of EMF did not affect BMSC functions compared to the groups without exposure to EMF. All in all, the magnetic PGS nanocomposites could be a potential candidate in future bone and cartilage tissue engineering applications. With the preservation of magnetic response abilities, the PGS nanocomposites are able to be remote manipulated by external magnetic field without side effects to BMSC functions.

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