ChemPubSoc Europe

DOI: 10.1002/cphc.201701294

CHEMPHYSCHEM Minireviews

Special Issue

Adaptive Materials Based on Iron Oxide Nanoparticles for Bone Regeneration

Yan Li,[a] Dewen Ye,[a] Mingxi Li,[a] Ming Ma,[a] and Ning Gu*[a]

The paper provides a brief overview of the use of iron oxide nanoparticles (IONPs) in the areas of bone regenerative medicine. Reconstruction of bone defects caused by trauma, non-union, and bone tumor excision, still faces many challenges despite the intense investigations and advancement in bone-tissue engineering and bone regeneration over the past decades. IONPs have promising prospects in this field due to their controlled responsive characteristics in specific external magnetic fields and have been of great interest during the last few years. This Minireview aims to summarize the relevant progress and describes the following five aspects: (i) The general introduction of IONPs, with a focus on the magnetic properties as the base of application; (ii) using IONPs as tools to study and control stem cells for better treatment efficacy in stem-cell-based bone defect repair; (iii) the use of IONPs and their complexes in the delivery of therapeutic agents, including chemical drug molecules, growth factors, and genetic materials, to promote osteogenesis-related cell function and differentiation, healthy bone tissue growth, and functional reconstruction; (iv) magneto-mechanical actuation in the regulation of cells distribution, mechano-transduction membrane receptors activation, and mechanosensitive signaling pathways regulation, and (v) fabrication, characteristics, and in vitro and in vivo osteogenic effects of magnetic composite bone scaffolds. Ongoing prospects are also discussed.

1. Introduction

Due to its excellent biocompatibility and unique magnetic properties, iron oxide nanoparticles (IONPs), mainly magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3), have attracted researchers' considerable attention, and have been extensively studied for biomedical applications.[1] With the applied magnetic field, introduced IONPS in biological systems is known to produce required effects. For example, they are commonly used engineered biocompatible nanoparticles, and are FDA-approved as contrast agents,[2] iron replacement therapies,[3] and tumor therapies using local tissue hyperthermia.[4] This approach creates flexible, controllable and well-defined methods for the remote control of biochemical processed both in vitro and in vivo. At the same time, it has been noticeable that more and more cross-disciplinary teams are being formed to work in specific areas towards chosen targets of known clinical need. The close collaborations between medics, clinicians, life scientists, pharmacologists, physical scientists and engineers have enabled the additional uses for IONPs in biomagnetism. With the development of stem cell research and regenerative medicine, the possible applications of IONPs in regenerative medicine, especially bone tissue engineering and bone regeneration, have been preclinical studied.[5] Increasing research shows that magnetic fields and magnetic responsive scaffolds can promote bone repair and regeneration. For that reason, this short review intends to summarize some of the progress achieved in the development of adaptive materials based on iron oxide nanoparticles for bone regeneration. The scope of this minireview will include general characteristics of IONPs, IONPs-based stem cell therapies in the field of bone regeneration, magnetic-based drug delivery for health bone growth, magneto-mechanical actuation in bone tissue engineering and bone regeneration, IONPs based composites bone scaffolds for bone repair. The possible underlying mechanisms and perspectives of IONPs based materials in the bone regeneration are also included.

2. General Characteristics of IONPs

The structure and properties of IONPs are the basis of their biomedical applications. There are several criteria that should be considered when designing a nanoparticle system for use in biomedicine. The most obvious criterion for biomedical applications is safety and lack of intrinsic toxicity. IONPs are preferred for biological application because it is a naturally occurring metal in humans (e.g., ferritin in myoglobin and hemoglobin), allowing preexisting metabolic pathways to process the remaining iron from nanoparticles. However, bare IONPs may be toxic because their surfaces are chemical reactive and they are easy to aggregate together. The coating of IONPs serves important roles in reducing iron oxide oxidation, preventing aggregation and agglomeration of extracellular nanoparticles, and increasing biocompatibility.[1b] In addition, for different purpose, improved targeting to special tissue or cell, increased tracking duration, limited nonspecific cell interactions, and improved localization are also factors to be considered.[6] All the properties mentioned above are also given by the surface modification layer of the IONPs. Therefore, the

[a] Dr. Y. Li, D. Ye, M. Li, Dr. M. Ma, Prof. N. Gu
Southeast University, State Key Laboratory of Bioelectronics, Jiangsu Key Laboratory of Biomaterials and Devices, School of Biological Science and Medical Engineering, Sipailou 2, 210096 Nanjing (China)
E-mail: guning@seu.edu.cn

Special Issue An invited contribution to a Special Issue on Smart Materials

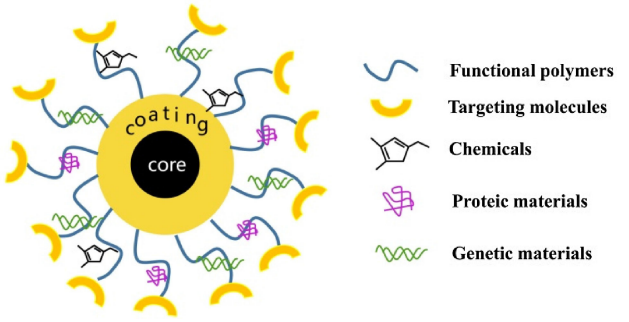

Figure 1. Simplified schematic of IONPs. IONPs are nanoparticles with an iron oxide (maghemite or magnetite) core with surface coating. The coating may be two or more layers with functional molecules, e.g., polymers for better stability and biocompatibility; Targeting molecular such as ligands, antibodies, and aptamers for specific targeting; chemicals for diseases treatment; proteic/genetic materials for regulation of cell biochemical processes.

general IONPS are composed of an iron oxide core, which is enveloped by an organic or inorganic coating. The first coating layer may be modified with the second or more layers to provide specific functions. The organic matrix coating can be citrate, dextran, polyethylene glycol, chitosan, polyethyleneimine, phospholipids, or copolymers.[7] In addition to organic matrix, inorganic matrixes such as silica,[8] gold,[9] and calcium phosphate[10] could also be available for the synthesis of such core-shell structure. And the coating layers can also be chemically or physically handled for conjugation with targeting molecules (i.e., ligands, antibodies, and aptamers) to specific binding with receptors, and loading therapeutic agents including drug chemical molecules, proteic and genetic materials for certain medical purpose.[11] Figure 1 illustrates simplified schematic of IONPs.

The magnetic properties of IONPs are crucial for their applications. There is no doubt that IONPs will respond to an applied magnetic field. Even without the application of an external magnetic field, from the physics standpoint, the iron oxide nanoparticles themselves can be considered a single magnetic domain to provide a magnetic field at a nanoscale. The interaction between the IONPs and the applied magnetic field, as well as the interaction between nanoparticles, both will produce energy changes in the system. This energy will clearly define the potential effects of IONPs in the biological context. As described in the previous review,[12] the magnetic field and accompanying IONPs can affect biochemical processes through several theoretically justified and experimentally validated mechanisms.[13] One extensively studied and well-established mechanism is based on spintronics.[14] This has greatly promoted the progress of medical technology. For example, the invention of magnetic resonance imaging (MRI) and its application in medical diagnosis.[15] The other two mechanisms of utilizing the acquired energy, magnetic hyperthermia using alternating magnetic fields (AMF)[16] and magneto-mechanical actuation using gradient magnetic fields,[17] have also been clearly analyzed in reviews elsewhere. Figure 2 shows the applications of IONPs under the applied magnetic field through three different routes, with a focus on the field of bone regeneration.

To understand these mechanisms better for guiding the synthesis of IONPs and their biomedical application, we need to be aware of some of the fundamental concepts of magnetism, which will be recalled briefly here. More details can be found in one of the many excellent textbooks on magnetism. All

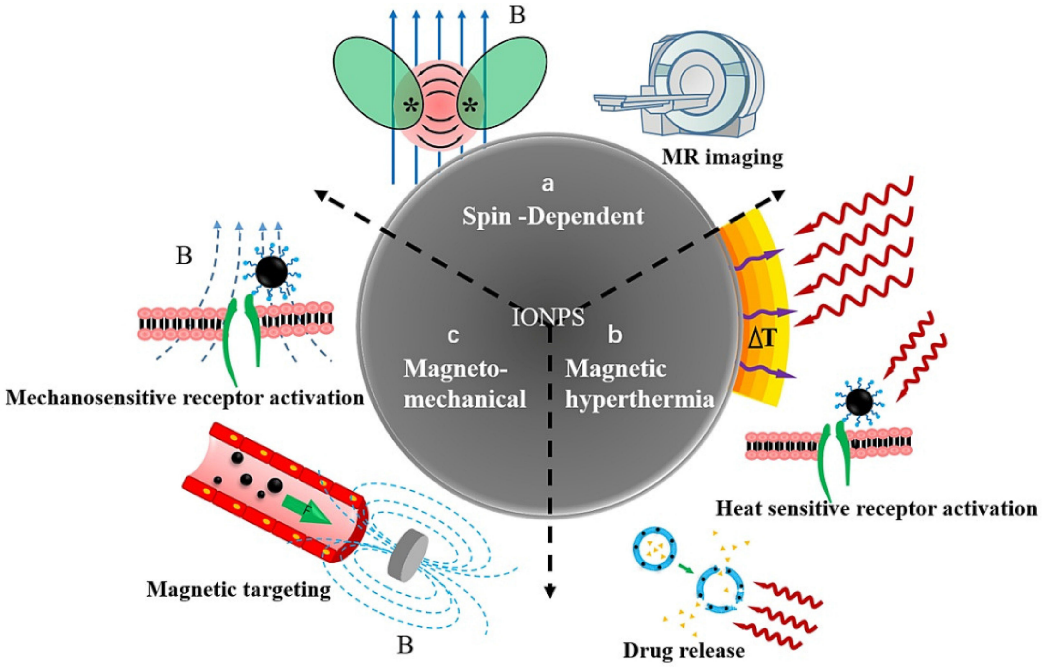

Figure 2. Three different ways for IONPs applications under a magnetic field. a) Spin-dependent for in vivo tracking by MRI, b) magnetic hyperthermia by alternating magnetic field for drug release from heat-sensitive vesicle and heat-sensitive receptor activation, c) magneto-mechanical actuation by gradient magnetic field for magnetic targeting and mechanosensitive receptor activation.

materials are magnetic to some extent with their response depending on their atomic structure and temperature. They may be conveniently classified in terms of their volumetric magnetic susceptibility, χ, where:

$$M = \chi H \quad (1)$$

describe the magnetization induced in a material by H (magnetic field strength).[17] According to the value of χ, the materials are classified as paramagnets, diamagnets, ferromagnets, ferrimagnets and antiferromagnets. Fe_3O_4 and γ-Fe_2O_3 are ferrimagnets. The characteristic shape of the *M–H* curve is sigmoidal, with M approaching a saturation value at the large value of H. An irreversible hysteresis often occurs and gives rise to open *M–H* curve, called hysteresis loops. The shapes of these loops are determined in part by particle size. An important phenomenon[18] is that ferromagnetic and ferrimagnetic (FM) materials exhibit high magnetization with a low applied magnetic field and have a remnant magnetization with the elimination of the applied magnetic field, while small IONPs (usually smaller than 20 nm diameter) exhibit superparamagnetism, in which the nanoparticles saturate with relatively high magnetization with a low applied magnetic field, but have no net magnetization with the removal of an applied magnetic field.

FM particles possess hysteretic properties when exposed to a time varying magnetic field, which gives rise to magnetically induced heating. However, the superparamagnetic IONPs' magnetization relaxes back to zero due to the ambient thermal energy of the around environment when the external magnetic field is removed. The relaxations are referred to Neel and Brown relaxations. Neel relaxation is caused by the movement of the magnetic moments relative to the crystal lattice structure of the IONPs. While Brown relaxation involves the movement of the IONPs relative to the surrounding medium.[19] These processes result in the dissipation of the magnetic energy and heat generation from IONPs, which is characterized by the magnetic parameters such as relaxation time, and specific absorption rate (SAR). This is the physical basis of the heating of IONPs by AMF.

To understand how a magnetic field may be used to manipulate IONPs, we need to recall some elements of vector field theory. If a magnetic particle is placed in a magnetic field of strength H, the magnetic force acting on a particle:

$$F_m = \mu_0 \chi_{eff} V_p (H \cdot \nabla) H \quad (2)$$

when it is unsaturated magnetization, and

$$F_m = \mu_0 V_p (M_{ps} \cdot \nabla) H \quad (3)$$

when it is unsaturated magnetization. Where μ_0 is the permeability of free space, $\chi_{eff}$is the effective magnetization coefficient of the particle, V_p is the volume of the particle, M_{ps} is the saturation magnetization of the particle, and the magnetic induction $B = \mu_0(H + M)$, which can be used to judge whether the particles are saturated magnetized.

It is usually recognized that a magnetic field gradient is required to exert a force at a distance; a uniform field gives rise to a torque, but no translational action.[17,20] However, regardless of the type of magnetic field applied, IONPs may be subject to magnetic forces. This is because, even if the applied external magnetic field is uniform, the presence of magnetic IONPs in an otherwise magnetically disordered environment (aqueous solution, cell or tissue) will induce a very localized effect of the external magnetic field, as described above, which results in the appearance of a gradient magnetic field. Golovin et al. has presented the models of the magneto-mechanical effects of an AMF and discussed the optimal characteristics of the magnetic nanoparticles and an AMF for effective magneto-mechanical actuation of single molecule responses in biological and bio-inspired systems in his published review.[12]

Fe_3O_4 and γ-Fe_2O_3 present FM behavior and the main difference between these two structures is that while Fe_3O_4 has Fe^{2+} and Fe^{3+} cations, γ-Fe_2O_3 has only Fe^{3+} cations and vacancies in their sub-lattices.[21] Identification of magnetite and maghemite is a very difficult task because of their identical structure and their small variations in lattice parameters.[22] Reaching to the nanometer scale particle size, interactions become effective factors in the formation of magnetic property.[23] It could be seen from the above description, the magnetic properties of IONPs and their responses to a magnetic field are very strongly correlated to their core size. And this has been also confirmed by experiment.[24] Taking SAR as an example, our previous studies[25] have shown that the SAR of IONPs decreases as the nanoparticle diameter increases above 50 nm. And the heating capacity of IONPs in a radio frequency (RF) alternating current (AC) fields is also very strongly correlated with nanoparticle size, with the more efficient heating occurring with particles of 14–16 nm in diameter.

Besides size, the morphology and structure will also affect the magnetic parameters.[26] Anisometric particles when compared with isometric nanoparticles are likely to behave quite differently both magnetically and when interacting with biological entities[27] and this is expected to influence the relaxation times. Moreover, the IONPs local concentration and aggregation state (particles are easy to aggregate at the nanoscale) can also affect the heating efficiency of IONPs for application in magnetic hyperthermia.[28] In addition, researchers have shown that the saturation magnetization of magnetic nanoparticles is also strongly depend on the particle size and shape,[29] agglomeration and surface chemistry of the material,[30] which are determined by the synthesis route.

In addition to the magnetic properties, other aspects such as their comparable size to the biological substances also affect the biomedical application of IONPs. The different strategies by which IONPs can be used for bone regeneration are illustrated in Figure 3. Hence, it should be noted that particle size, morphology and structure do not only affect the magnetic properties of IONPs, but also play an important role in the interaction between particles and biological systems. For example, these factors can affect the biodistribution and blood circulation duration of IONPs.[6,31] Therefore, specific cell or

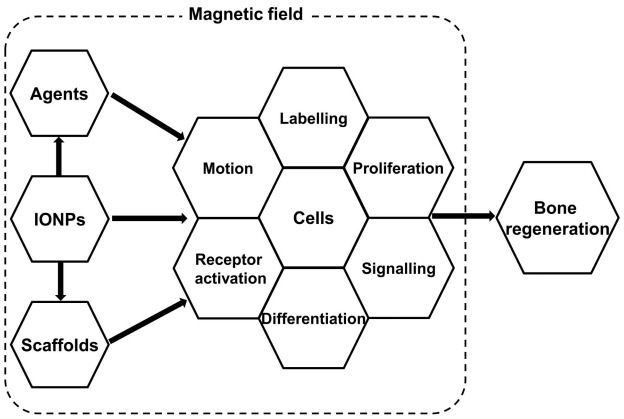

Figure 3. IONPs-based approaches for bone regeneration. IONPs can affect the behavior and function of bone forming cells (labeling, motion, proliferation, receptor activation, signaling, differentiation, etc.) through different ways: delivery of therapeutic agents, magnetizing composite scaffolds and themselves to promote bone regeneration. The approaches might be associated with the use of an external magnetic field.

tissue targeting, the rate of cellular uptake, functional molecules or drugs, and other factors should be taken into account in the construction of IONPs. The IONPs core synthesis technique and the subsequent coating and functional process should allow for good control over particle size, size distribution, shape, porosity, and surface charges.

However, few studies have been devoted to accounting for the influence of the above two structural phases (Fe_3O_4 and γ-Fe_2O_3) on the magnetic properties and the subsequent promotion of bone regeneration. Here we try to summarize the application information of different types of IONPs for bone tissue engineering and bone regeneration (Table 1). There remains a lack of standardization for the application of IONPs in the field of bone regeneration. However, the use of such therapies to facilitate bone cells growth and bone tissue healing appears quite promising. More details will be introduced in the following text.

3. IONPs-Based Stem Cell Therapies for Bone Regeneration

It has been a major clinical challenge for the repair of bone defects caused by fracture, non-union, and bone tumor excision. Treatments employed for bone regeneration are based on the use of cells, matrix materials and growth factors. Cell treatment with stem cells may provide a promising strategy for bone regeneration and healing, especially for the repair of large bone defects.[42] The application of stem cells includes direct cells injection or matrix associated stem cell implantation. Although great success has been achieved with stem cells in promoting bone regeneration in animal models, these traditional therapies by transplantation of stem cells did not demonstrate the success initially envisaged, and there are still a number of gaps in our knowledge which need to be addressed before this treatment can be widely applied in clinical patients. For example, effective retention of transplanted cells is vital to

Table 1. Example studies using IONPs in bone regeneration.

Method	IONP	Magnetic field	Cell type	Animal model	Ref.
enhancing osteogenic differentiation by magnetized composite	γ-Fe_2O_3	pulsed electromagnetic field	rabbit bone marrow MSCs	no application	[32]
accelerating new bone tissue formation by magnetized composite	γ-Fe_2O_3, ~13 nm,0.049 emu/g[a]	permanent magnets	no application	rabbit lumbar transverse defects	[33]
enhancing cell growth by magnetized composite	γ-Fe_2O_3, ~13 nm	no application	mouse primary bone marrow cells	no application	[34]
enhancing cell growth by loading bFGF	Fe_3O_4,36.5 ± 0.7 nm	no application	SaOS-2 cells	no application	[35]
improving cell proliferation via activation of MAPK signaling pathway by magnetized composite	Fe_3O_4(56 emu/g[b]), 10 nm, 2 emu/g[a]	no application	MC3T3-E1 cells	no application	[36]
enhancing bone forming ability by magnetized composite	Fe_3O_4,12 ± 1.34 nm, 1.0 ~ 11.2 emu/g[a]	no application	rat bone marrow MSCs	rat radius segmental defects	[37]
promoting cell growth by magnetized composite	Fe_3O_4, ~100 nm, 0.004 ~ 0.8707 emu/g[a]	no application	MG-63 cells	no application	[38]
enhancing bone healing by magnetized composite	Fe_3O_4, < 50 nm for in situ nucleation, and 200 nm for infiltration	no application	no application	rabbit distal femoral epiphysis and tibial middiaphysis defects	[39]
increasing osteogenic potential in vitro and bone healing in vivo by magnetofection	Iron oxide,88 nm	magnet	human adipose-derived stromal cells	mouse calvarial defects	[40]
enhancing bone healing by Mag-TE	Fe_3O_4,10 nm	a cylindrical magnet for construction of MSC sheets, and an electromagnet for transplantation of MSC sheets	human bone marrow MSCs	rat crania defects	[41]

[a] Saturation magnetization value of magnetic composite scaffolds. [b] Saturation magnetization value of IONPs themselves.

the success of cell treatment. Hence, in vitro cell expansion with the maintaining of a stable phenotype, and in vivo reducing cell necrosis and loss from the defect sites should be concerned to obtain the large number of cells required for regeneration of defected bone tissues. Also, it is necessary for us to investigate the mechanism by which stem cells stimulate and mediate bone regeneration in vivo, and how to induce the osteogenic differentiation of stem cells both in vitro and in vivo.

Nanotechnology has been employed to fill these gaps. Nanoparticles including IONPs as tools to study and control stem cells has been tried over the years.[43] The effects of IONPs usually combined with the applied magnetic field on almost all the cell behaviors, such as cell adhesion and proliferation, cell motility and distribution, cell expansion, osteogenic/chondrogenic differentiation of stem cells, and deposition of an extracellular matrix (ECM) have been studied. And the subsequent in vivo tracking and monitoring of cells labelled with nanoparticles has also been tried. Here, we try to summarize example studies of IONPs in the promotion of bone regeneration by stem cell therapy. It is noticed that the osteogenic effects of bone forming cells (stem cells, osteoblast progenitor cells, and osteoblast cells) induced by therapeutic agents carried with IONPs-based materials will be described in section 4. And the motility and distribution of cells (in addition to stem cells, other bone forming cells were also involved), which is mainly actuated by magnetic force, not including the homing of cells to the injury sites, will be discussed in section 5.

One application of IONPs in stem cell therapy for bone regeneration is the magnetic targeting of stem cells to the deserved sites, known as magnetic homing of stem cells. An in vivo study reported by Oshima et al. used an external magnetic targeting system to attract rabbit bone marrow-derived mesenchymal stromal cells (BMSCs).[44] This technique significantly facilitated the infiltration of ferumoxide-labelled cells into porous hydroxyapatite ceramic implanted in a rabbit ulnar defect and significantly contributed to the enhancement of bone formation even in the chronic phase. In another study, a magnetic targeting system for repair of severe chronic osteochondral defects using magnetically labeled mesenchymal stem cells (MSCs), with the aid of an external magnetic device, was investigated.[45] Complete repair of the severe chronic defect including cartilage and subchondral bone was confirmed with transplantation of 2×10^5 MSCs.

Ito et al. reported a new methodology of MSCs expansion using magnetite cationic liposomes (MCLs) with Fe_3O_4 nanoparticles as cores and a cylindrical neodymium magnet which provide magnetic force vertical to the shaking culture dish. The MSCs magnetically labeled by MCLs were enriched and then cultured, resulting in much higher density (seeding density of 1000 cells/cm^2) than in ordinary culture (seeding density of 18 cells/cm^2). And the high seeding density caused an increase in the number of cells.[46] Thereafter, magnetic beads conjugated with anti-rat CD44 mouse monoclonal antibodies has been used to accumulated MSCs effectively. And the further investigation for osteogenic differentiation of MSCs coupled with magnetic beads in vitro clearly demonstrated the possibility of this system for bone regeneration.[47]

Another treatment to enhance bone regeneration using stem cell therapy is promoting the osteogenic potential of stem cells. Both pre-differentiation in vitro and enhanced differentiation in vivo are available. The ability of IONPs to delivery proteic/genetic via an intracellular route presents an excellent tool to control the growth and differentiation of stem cells. The details are discussed in section 4 of this article.

In addition to inducing osteogenic differentiation of stem cells, another approach has been examined being the transplantation of "MSCs pre-differentiated in vitro into cartilage-forming chondrocytes" into bone defects, in brief, representing the route of "endochondral ossification (indirect bone formation)" instead of the "intramembranous ossification (direct bone formation)" route. The healing of a massive 15 mm femur defect (approximately 50% of the rat femur shaft length) provided a sound foundation for potential clinical application of this technique.[42e] And this is a paradigm shift of stem cell therapy for bone regeneration.

Cell migration, distribution, viability, differentiation, and fate following injection into the area of injury all play crucial roles in treatment efficacy of stem cell therapy. Understanding of these parameters allows the optimization of cell choice, delivery route, and dosage for therapy and advances cell based therapy for specific clinical uses. To address this issue, researchers have kept searching for tools that allow real-time, quantitative, and long-term monitoring of cell delivery and behavior in vivo, also known as cell tracking. IONPs, as a NMR contrast agent, can be used for cell tracking by MRI. In a study, IONPs labeled BMSCs were implanted into skull defects of Sprague-Dawley rats, the labeled cells were effectively tracked in vivo by MRI, and histological examination and statistical analysis indicated that IONPs labeling did not affect the viability or differentiation ability of BMSCs.[48] IONPs-based MSCs-tracking was also attempted to implement in a sheep ovine model of tendonitis and the labeled cells remained detectable by MRI at 7 days.[49] Furthermore, Nedopil et al. compared the MR signal characteristics of FDA approved ferumoxides nanoparticles labeled apoptotic and viable hMSCs in matrix associated stem cell implants.[50] The untreated and apoptosis induced hMSCs in an agarose scaffold were implanted into cartilage defects of porcine patellae specimens and underwent MR imaging at 7T. The results demonstrated that apoptosis induction resulted in a significant decline of T2-signal, which means MRI could be successfully used for noninvasive diagnosis of stem cell efflux and necrosis. This approach for the diagnosis of apoptosis in transplanting cells in vivo could significantly improve our ability to identify favorable tissue engineering constructs, improve implant surveillance, and ultimately help to optimize our efforts to restore the functions of damage tissue.

In addition to the above-mentioned strategies to promoting bone regeneration by controlling stem cells, IONPs themselves, especially when combined with the stimulation of an external magnetic field, would induce the osteogenic differentiation of stem cells. This has been confirmed in our previous studies. We prepared the polyglucose sorbitol carboxymethyl-ether (PSC)

coated IONPs and explored their effects on hBMSCs in vitro. The results showed that our as-prepared IONPs were structurally stable in hMSCs and promoted osteogenic differentiation of hMSCs. Systematic analyses by use of gene microarray assay and bioinformatics analysis revealed that gene expression was widely regulated and classical mitogen-activated protein kinase (MAPK) signal pathway was activated by IONPs treatment, and downstream genes of this pathway were regulated to promote osteogenic differentiation.[51] In addition, IONPs treatment upregulated long noncoding RNA INZEB2, which is indispensable for maintaining osteogenesis by regulating ZEB2 expression and BMP/Smads pathway.[52]

Jiang et al. also reported that uptake of Fe_3O_4/BSA particles enhanced significantly osteogenic differentiation of MSCs under a static magnetic field, as evidenced by elevated ALP activity, calcium deposition, and expressions of collagen type and osteocalcium at both mRNA and protein levels,[53] which was consistent with our research.

Suspensions of small IONPs can form linear chain-like or fibrous aggregates in the presence of an applied external magnetic field. Actually, a number of studies on magnetic nanoparticles assembly in the external field have been carried out, and many researchers keep interest in it.[54] Our group has also published a few papers which reported the assembly of nanoparticles including IONPs under the external magnetic field.[55] Moreover, we also studied the effect of the assembled IONPs on MSCs in vitro.[56] In our study, the stripe-like IONPs assemblies were produced under the external magnetic field combined with solvent evaporation. The assembled patterns were mainly determined by the magnetization of particles (actually, the clusters of nanoparticles) and the particle concentration, while little affected by the strength of the external magnetic field, which was revealed by theory analysis and confirmed by morphological characterization using scanning electron microscope (SEM). The assembly IONPs could promote the differentiation of BMCs into osteoblasts. And the reason was thought to lie in the remnant magnetic interaction inside the assemblies which resulted from the magnetic field-directed assembly. Influence of the assemblies on the cells was realized by means of interface effect rather than the internalization effect. These results about the cellular regulation with nanoparticle-mediated magnetic effect will be favorable for the intensive investigation and extensive application of stem cell technology.

4. Magnetic-Based Drug Delivery for Bone Growth

Pharmaceutical species such as antibiotics to treatment infection, drugs for the treatment of bone disease, growth factors as well as microRNA or siRNA for enhancing the proliferation and differentiation of osteogenesis related cells are available for specific use in bone regenerative medicine to improve the treatment efficacy. However, these drugs are often not specifically targeted to the desired sites and, thus, lack an immediate directed therapeutic effect. Researchers keep paying attention to searching for optimal drug delivery systems for site-specific delivery of drug, which will minimize undesired, adverse effects and toxicities of the delivered drug, reduce the loss of drug efficacy, and improve the treatment effect. Using IONPs as vehicles would carry drug molecules effectively to target specific locations in the body with the application of an external magnetic field.

A simple and direct way to use magnetic targeting drug delivery is the drug molecules being suspended to the magnetic vehicle or dispersed on top of the magnetic nanoparticles. For example, poly-L-lactide co-glycolide (PLGA) dissolved in chloride was added to the capsaicin-dispersed magnetic fluid, and the product of biodegradable PLGA-coated capsaicin magnetic nanoparticle (PCMN) was achieved by solvent evaporation and freeze-drying.[57] The PCMN provided a sustained release of capsaicino which could be advanced for site specific pain therapeutics by manipulating the localization of the magnetic particles to the desired sites under the application of an external magnetic field. It is worth noting that capsaicin is an agonist of transient receptor potential family, vanilloid type 1 (TRPV1) channel proteins. The conclusion showed in the study was also obtained by an increased efficacy to activate TRPV1 channel protein, which was confirmed by the intracellular Ca^2 imaging. It has been reported that TRPV5/6, which belongs to the same TRPV cation channels family, is vital for bone formation.[58] Thus, it is believable that this kind of complex may be used in the field of bone regeneration.

Besides magnetic targeting, magnetic heating has also been used to achieve controlled release of therapeutic agents from thermally responsive drug carriers. In a study, bisphosphonate (Bis) was conjugated to IONPs coated with dextran (Dex) to get Bis/Dex/Fe_3O_4 nanoparticles.[59] The thermolysis of as-prepared particles by a RF system (42 kHz and 450 A) resulted in a sufficient destroy of osteoclasts swallowing with Bis/Dex/Fe_3O_4 nanoparticles. At the same time, the synthesized IONPs could be indeed magnetic resonance imaging contrast agents too.

A number of materials with embedded IONPs have been proposed for controlled drug release. For example, magneto-liposomes obtained by embedded IONPs into or attaching IONPs to lipid membranes of liposomes have attracted much attention. When the external AMF was applied, the generated heat would disrupt the membranes and thereby release the drug encapsulated in the liposomes. In particular, this has been well addressed for non-bone application.[44,60]

Actually, complexes were usually used as carriers for the controlled release of the drug in the areas of bone regenerative medicine. In addition to carrying the drug, the complex should also have good osteoconductivity. From the theory standpoint, almost all the magnetic scaffolds for bone regeneration can be used as drug carriers. Farzin et al. prepared a multifunctional magnetic Hardstonite (HT) scaffold which aimed at regeneration of large-bone defects caused by malignant bone tumors through a combination of hyperthermia, local drug delivery and osteoconductivity.[61]

Drugs can be carried by IONPs-based composites to treat bone regeneration related diseases. Implant-associated infection is a serious problem in orthopedic surgery. The infection around a bone graft will result in surgery failure and bring huge suffering to the patients. Compared to infusing, administering antibiotics at the sites of bone defect and delivering locally in a controlled manner will enhance the accessibility of drugs to the infection site in bone tissue, particularly in necrotic or avascular tissues. Many researchers focus on introducing local drug delivery systems in bone reconstruction surgeries. For example, gentamicin has been loaded in a multifunctional magnetic mesoporous bioactive glass (MMBG).[62] The Fe_3O_4 nanoparticles in MMBG have improved sustained release of gentamicin, which was beneficial to minimize bacterial adhesion and prevent biofilm formation. Magnetic mesoporous carbonated hydroxapatite microspheres (MHMs) have also been fabricated for loading gentamicin by the same investigators.[62] In addition to treat infection, the Fe_3O_4 nanoparticles in the composites would promote the cell adhesion, proliferation, and osteogenic differentiation of hBMSCs, which suggested that MMBG and MHMs could be used as good drug carriers for the treatment of complicated bone defects.

Nanocomposite systems (HA/MWCNT/Fe_3O_4) obtained using magnetic multi-walled carbon nanotubes as fillers for hydroxyapatie (HA) either decorated or not decorated with Fe_3O_4 nanoparticles by a deposition method have been synthesized and doped with clodronate.[63] The doped clodronate could be released in vitro from the systems, and inhibit the formation of osteoclast which was confirmed by preosteoclastic RAW264.7 cells tests. The reported nanocomposites could be a biocompatible magnetic drug delivery system and could represent a useful multimodal platform for applications in bone tissue engineering.

Grown factors play key roles in the bone regeneration. Fan et al. have developed a magnetic biopolymer based nanogels chitosan and heparin by specific nucleobase pairing between thymine and adenine via the hydrogen bonding for vectoring delivery of bone morphogenetic protein 2 (BMP-2).[64] The encapsulation of IONPs gave the magnetic biopolymer nanogels magnetic responsibility. The vectoring delivery of incorporated BMP-2 through binding with heparin could be easily controlled by the external magnetic field. In vitro tests demonstrated that the biopolymer nanogel could efficiently promote the viability of MG-63 cells, in particular, under a magnetic field, which suggested a promising future of the magnetic nanogel for cartilage and bone regeneration applications. In another study, Magnetic silk fibroin e-gel scaffolds has been developed, basic fibroblast growth factor (bFGF) was conjugated physically to human serum albumin coated Fe_3O_4 nanoparticles blended in the gel and had an inductive effect on SaOS-2 cells viability. Moreover, bFGF loaded silk fibroin e-gels showed significantly enhanced alkaline phosphatase activity and calcium deposited activity of SaOS-2 cells cultured on them.[35]

In all the applications discussed above, researches focus on efficient loading of the drugs or biomacromolecules into the IONPs-based carrier as well as safe and control delivery of the loaded drugs to the bone defects. Besides drugs delivery to the target organs and tissues, intracellular delivery of drugs is of great interest. Drug delivery to the internal cells and even the specific organelles makes it possible to fine regulation of the cell behaviors. Advances in nanotechnology have led to the development of intracellular drug delivery. An important strategy[43] is using nanomaterials as carriers for the intracellular delivery of therapeutic agents, including proteins, growth factors, small chemicals, and DNA/RNA materials, which would be benefit for therapy. Magnetic IONPs exhibit specific advantages especially when combined with the external magnetic field in this field. This not only depends on the interactions between nanoparticles and cells, but is mainly related to IONPs' magnetic responses to the external magnetic field.

For example, delivering genes to enhance cells function and promote the bone formation activity of stem cells has been explored. Highly efficient methods of human gene therapies have been developed based on viral vectors, however, the viral systems may have some potential drawbacks. For example, adenoviral vectors might induce unexpected inflammatory responses, thereby decreasing its therapeutic efficacy.[65] And retroviral vectors might cause random integration of viral DNA into the host genome, which poses a risk of neoplastic transformation. Nonviral vectors such as liposomes were lack of the above drawbacks but have significantly low transduction efficiency. Magnetofection is a new method for gen transfer that involves the use of magnetic force and plasmid DNA (pDNA)/magnetic bead complex, and it has been developed for enhancing delivery of gene vectors to target cells.[66]

In another study, iron oxide cores were sequentially coated with branched polyethyleneimine, minicircle plasmid encoding green fluorescent protein and Bcl-2, and ply-β-amino ester. To evaluate the ability of IONPs to transfect cells, fully layered IONPs were then cultured with adipose-derived stromal cells (ASCs) and were seen to have higher expression of GFP (31%) compared with cells nucleofected with the same GFP/Bcl-2 plasmid. Furthermore, fully layered IONPs were integrated with HA-PLGA scaffold and the prefabricated scaffold combined with the seeded ASCs was placed into critical-size mouse calvarial defects. The results showed that magnetofection had an efficiency rate of 30% and in turn resulted in significantly more healing compared with control group and nucleofected group, which indicated an effective technique for in situ postimplant temporospatial control of cell transfection to augment bone regeneration.[40]

MicroRNAs, key regulators of gene expression on the post-transcriptional level, have also been delivered into hMSCs with the help of a magnetic non-viral vector based on cationic polymer polyethylenimine (PEI) bound to IONPs.[67] Optimized magnetic complexes caused high microRNA-335 uptake rates (yielded ~75%). The results would be helpful to the virus-free introduction of therapeutic microRNA as well as other nucleic acids in vivo.

5. Magneto-Mechanical Actuation in Bone Tissue Engineering and Bone Regeneration

As described in section 2, magnetic field can actuate mechanical motion of the IONPs, and this in turn can affect the cells, subcellular structures and biomacromolecules to which such nanoparticles are attached. The biological application of magneto-mechanical actuation has long been studied. One aspect of this is magneto-mechanical actuation for tissue engineering and regenerative medicine, which has emerged over the past decades.

One of the important applications in tissue engineering is to control the distribution of cells in tissue engineering complex. Tissue engineering is a method of trying to grow functional tissue in damaged sites. This approach generally includes three elements: seed cells, growth factors, and three-dimensional (3D) biodegradable scaffolds. The construction of tissue engineering complex is important for the promotion of tissue regeneration or healing. The main problem is that cells normally stay on the surface of these structures and do not enter the scaffold. Using magneto-mechanical actuation to drive cells to the center of the 3D scaffold, or control the formation of desired cell structures may be achieved.

Honda and co-workers contributed much in this field. For example, they reported a cell-seeding technique using magnetic force, which termed Mag-seeding. Porcine decellularized common carotid artery (dCCA) was used as one of the most promising scaffolds. When the dCCA was immersed into a suspension of magnetically labeled cells, the cylindrical magnet inserted into the lumen of dCCA made almost all the cells attach onto the dCCA. And the cell-seeding efficiency by Mag-seeding was enhanced when the cellular uptake of IONPs increased.[68] In another study by Sasaki et al., IONPs coated with chitosan were developed to enhance human osteoblasts invasion into the depth of the 3D scaffolds, increase subsequent cell-cell interaction and shorten the period of cell proliferation using magnetic force.[69] This system is thought to be useful for the bone repair.

An in vitro reconstruction of three-dimensional tissues without the use of scaffolds may be an alternative strategy for tissue engineering and regenerative medicine. A series of studies were also reported by Honda H and his collaborators. They developed a cell-manipulation technique using functionalized magnetite nanoparticles and magnetic force, which was designated "magnetic force-based tissue engineering (Mag-TE)".[70] Various types of cell sheets, such as MSCs sheet,[71] adipose-derived stem cell sheet,[72] dermal cell sheet,[73] human dermal fibroblast sheet,[73] human retinal pigment epithelial cell sheet,[74] multi-cellular sheet,[70] multilayered cell sheets,[75] and even tubular structures[76] were constructed using Mag-TE, which has greatly driven the process in tissue engineering. In regard to bone tissue engineering, human MSCs magnetically labeled with magnetite cationic liposomes (MCLs) were seeded onto an ultralow attachment culture surface, and a cylindrical neodymium magnet (4000 G) was placed on the reverse side. The MSCs could form multilayered sheet-like structures after a 24 h culture period. When the magnet was removed, the MSC sheets detached from the culture surface. By using an electromagnet, the MSC sheets were easily harvested. The harvested MSC sheets could maintain an in vitro ability to differentiate into osteoblasts, adipocytes, or chondrocytes and could enhance the formation of new bone tissue when the MSC sheets were transplanted into a 5 mm defect made in the cranial bone of nude rats.[41] Induced pluripotent stem cell sheets were also created by Mag-TE for reparative angiogenesis.[77] According to the studies in the above described publications, this approach of Mag-TE is versatile, exhibiting great promise for generating more complex cellular structures for potential bone tissue engineering applications. Figure 4 shows the basic roadmap of Mag-TE.

In another study, Souza et al. reported a three-dimensional tissue culture based on magnetic levitation of cells in the presence of a hydrogel consisting of gold, IONPs and filamentous bacteriophage.[78] The geometry of the cell mass can be manipulated by spatially controlling of the magnetic field to achieve a multicellular clustering of different cell types in co-culture.

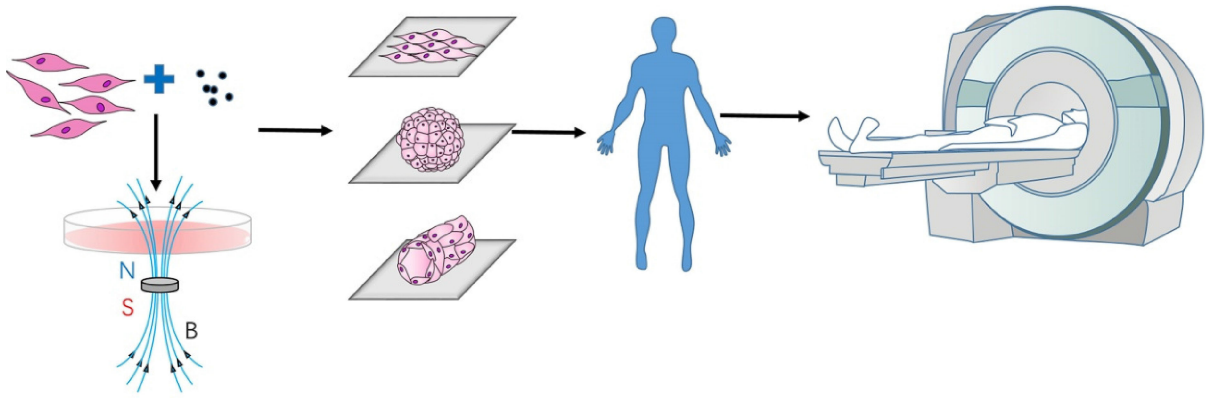

Figure 4. Cell labeling with IONPs for in vitro construction of cell population by magneto-mechanical actuation and in vivo tracking by MRI. When labeling with IONPs, cells can be actuated to construct specific structures, such as sheets, tubes, and spherical clusters. When the number of IONPs are enough for detection by MRI, the cells can be monitored in vivo.

It could be found out that in all studies described above, cell manipulation depended on the attachment of IONPs to the cells or cellular uptake of IONPs. The magnetic-mechanical actuation of nanoparticles by external magnetic field causes a pulling and shear stress in cells attaching or containing with IONPs, which allows cell manipulation. A very interesting study by Krebs et al. developed a new method for the magnetic manipulation of cells using a system that utilized the capacity of magnetophoresis to organize cells while avoiding the need for the attachment of magnetic materials onto the cells or cellular uptake of the particles.[79] Cells were immersed in a biological compatible ferrofluid consisting of suspension of BSA-passivated Fe_3O_4 nanoparticles with the dimensions being in the range of ~10–20 nm which could induce an effective magnetization of the extracellular fluid, and they would exhibit the field characteristics of a point dipole. The magnetic permeability difference between the cells and the surrounding fluid generated a magnetic adhesion energy, which drove the organization of cells into linear, oriented structures under uniform magnetic fields through negative magnetophoresis. The dimensions of the cellular chains were found to depend upon magnetic field exposure time and nanoparticle concentration. The linear cell assemblies were stable after removal of the magnetic field and ferrofluid, and the cells could be able to adhere to standard tissue culture surfaces and could then be further cultured for cells studies or tissue regeneration experiments. This cell assembly approach holds much promise for bone tissue engineering research.

Earlier studies in magneto-mechanical actuation relied primarily on relatively non-specific binding, or no binding, of larger (might micrometer-size) particles. Recently, work has focused on targeting specific ion channels and cell membrane receptors to initiate controlled responses by the cell. As we know, the realization of magneto-mechanical actuation of cells lies on the generation of magnetic force and its stress stimulation to the cells. In general, the mechanical stimulation can guide the development and function of cells through changes in gene expression. This is achieved by the process of mechanotransduction, which is how cells convert physical force into a biochemical signal. The process starts with the sense of mechanical cues by mechanoreceptors, which lead to changes in protein kinase or phosphatase activity inside the cell, and ultimately forward the propagated signal to activate transcription factors that regulate the expression of target genes. Figure 5 shows this process.

However, it is difficult to scrutinize the response of a single type of receptor because the response of a cell to applied force is not always straight forward. When force is applied at the macroscale, the whole structure of the cell is distorted that leads to the inadvertent activation of other receptors. IONPs can overcome the above experimental difficulties with macroscopic approaches. It is worth noting that the size of IONPs is similar to or even smaller than subcellular structures and biomacromoleculars, which makes it possible to precision manipulate and activate individual surface receptors such as ion channels on specific cells within a culture. Magnetic twisting cytometry and magnetic tweezer have been developed on this

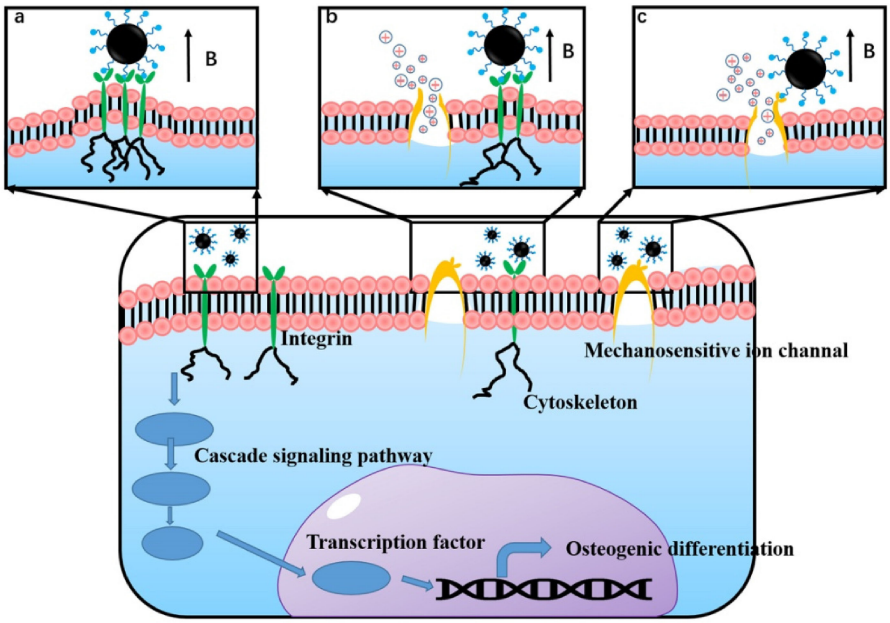

Figure 5. Process of mechanotransduction mediated by magneto-mechanical actuation. The process starts with the sense of mechanical cues by mechanoreceptors, which lead to changes in protein kinase or phosphatase activity inside the cell, and ultimately forward the propagated signal to activate transcription factors that regulate the expression of target genes. There are three ways to sense the mechanical cues. a) IONPs coated with RGD molecules attach to integrin receptors on the cell membrane. The receptors are linked to actin filaments. b) Mechanosensitive ion-channel activation: IONPs also bound to integrin receptors, the subjected magnetic force deforms the cell membrane and activate adjacent mechanosensitive ion channel. c) Targeted mechanosensitive ion-channel activation: IONPs are attached to a mechanosensitive ion channel through an antibody, the ion channel is directly opened by subjected force.

basis. IONPs can offer more control at the nanoscale because the strength, direction, and location of the magnetic force can be readily manipulated by the placement of the magnetic fields and the ligand coating on the IONPs. Nanomagnetic actuation has been proposed for this technique and it can be applied to several areas of biology and biomedical science: investigations of cell mechanical properties and mechanosensitive ion channel signaling pathways, targeted activation of specific ion channels, construction of 'biochips' and mechanical conditioning of cells for regenerative medicine applications.[20] A minireview by Sniadeki has been reported to summarize the application of nanomagnetic actuation technique in the field of activating cell signal emphases on mechanotransduction receptors and mechanosensitive signaling pathways.[42a]

It is well known, in addition to biochemical signaling molecules, mechanical signaling plays a pivotal role in maintaining bone cell function and remodeling of the skeleton.[80] Mechanosensitive ion channels are critical not only for sensing mechanical cues but also for transmitting downstream signaling events within mesenchymal or skeletal progenitor cells, which may trigger differentiation pathways toward an osteogenic lineage, and ultimately lead to promoted production of specific proteins which is related to bone growth and regeneration. Accordingly, the applications of nanomagnetic actuation mentioned above may contribute greatly in the field of bone regenerative medicine.

As the predominant molecular transducers of force, integrins play an essential role in the signaling and structure of a cell. The extracellular portions of integrin bind to ligands in the matrix and support cell adhesion, whereas the intracellular domains associate with the cytoskeleton through focal adhesion proteins, which regulate cell survival, differentiation, migration, and mechanotransduction pathways. It is meaningful to investigate the biological effects of integrins manipulation with magnetic nanoparticles. Related studies on integrins receptors, stretch-activated ion channels, focal adhesions, and the cytoskeleton which are key players in activating cell signaling pathways involved in mechanotransduction were summarized in Sniadecki's review.[42a] For bone regeneration, an early work by Pommerenke reported that stimulation of integrin receptors using a magnetic drag force could induce an intracellular free calcium response.[81] Mechanical stress on the α 2 or β1 integrin subunits increased intracellular Ca^{2+} in the osteogenic cell line U-2 OS, whereas mechanical loading of the transferrin receptor had a significantly lower effect. However, this study has focused on short-term stimulation experiments. To investigate the long-term effects, actuation of human osteoblasts using 4.5 μm magnetic microparticles coated with arginine-glycine-aspartic acid (RGD) was attempted.[82] In this study, the intracellular Ca^{2+} concentration also changed by the applying static magnetic field (~56 mT; gradient ~4.0 mT/mm), which means the changes in the intracellular calcium signaling. The results indicate that magneto-mechanical stimulation offer a tool for applying controlled mechanical forces to osteoblasts, and can be used to stimulate the intracellular calcium signaling over prolonged periods of time (up to 21 days), which would be benefit for the bone tissue engineering.

To define key mechanotransducers underpinning the above applications of magnetic tagging, such as controlled differentiation of MSCs, the electrophysiological responses of hMSCs have been investigated.[83] The dynamic force of 6 pN was directly targeted to cell surface integrins by the RGD coated ferromagnetic microparticles. hMSCs demonstrated cell membrane hyperpolarization responses after the application of force, mediated by BK channels and intracellular calcium release.

In addition, a series of interesting studies by Dobson et al. developed several strategies based on magnetic actuation. They designed and manufactured a special magnetic force mechanical conditioning bioreactor in which AMF with an amplitude of up to 120 mT, gradient 11 T/m and frequencies from 0 to 1 Hz can be generated by the mechanically moved set of permanent magnets.[84] Using this bioreactor, magnetic Fe_3O_4 or CrO_2 micro- and nanoparticles ranging from 250 nm to 2.7 μm in diameter could selective activate mechanosensitive TREK-1 ion channels[85]. The activation effects depended on the joint action of several aspects: TREK-1 transfection in COS-7 cells, magnetic particles modification with anti-His antibodies or complexes of nickel and nitrolotriacetic acid (Ni-NTA) for targeting the 6 histidine (6-His) loop regions of TREK-1, and magneto-mechanical actuation produced by the combination of magnetic fields and magnetic particles. Responses were absent when particles were coated with RGD peptide that did not bind to TREK-1 or when magnetic fields were applied in the absence of magnetic particles.

TREK-1 ion channel is a tandem pore potassium channel, which is highly expressed in various cells. RT-PCR, western blotting and immunohistochemistry were used to confirm that human derived osteoblast and MG-63 cell expression TREK-1 mRNA and protein.[42d] It is believed that TREK-1 could potentially be performing a number of important roles in bone forming cells. This would be confirmed by the further studies. The activation of TREK-1 can induce osteogenic/chondrogenic differentiation of stem cells and bone formation.[42b,86] To investigate the effects of TREK-1 activation on the differentiation of HBMSCs, IONPs-based magnetic beads of 250 nm were modified with Anti-TREK-1 antibodies for targeting binding to TREK-1. HBMSCs labelled with the modified magnetic beads were cultured in monolayer or encapsulated into polysaccharide alginate/chitosan microcapsules, and were stimulated with an AMF for 1 h each alternate day at cyclic loading intervals (f = 1 Hz, 1–100 pN/particle). In the case of magnetic beads targeting TREK-1, after a short-term conditioning of 7 days, HBMSCs in monolayer demonstrated a significant increase in mRNA levels of Sox9, core binding factor alpha1 (Cbfa1), and osteopontin. In the 21 days of in vitro bioreactor experiments and in vivo implantation subcutaneously in mice, repeated stimulation resulted in enhanced proteoglycan and collagen synthesis, extracellular matrix production and elevated the expression of type-1 and type −2 collagen.[42b] The results suggested that osteogenic mechanosensitive receptor manipulation by nanomagnetic actuation can induce the differentiation of osteoprogenitor cell populations toward an osteogenic

lineage. This kind of cell manipulation strategy offer tremendous therapeutic opportunities in bone tissue repair.

Another study on the induction of osteogenic differentiation of HBMSCs by receptor-targeted, magneto-mechanical stimulation was subsequently reported by the same group.[87] In this study, specific two mechanical-sensitive cell membrane receptors, platelet-derived growth factor receptor α (PDGFRα) and integrin $\alpha_v\beta_3$ were selected as subject investigated. 250 nm IONPs differently modified with antibody to PDGFRα or integrin $\alpha_v\beta_3$ were prepared for targeting actuation. The results showed that, compared to stimulation of integrin $\alpha_v\beta_3$ and non-treated controls, magneto-mechanical stimulation of PDGFRα by the application of a static magnetic field (60 to 120 mT, gradient ~3.3 to 11.0 mT/mm) 1 h daily increased the mineral-to-matrix ratio after 3 weeks of stimulation in the vicinity of the cells. Moreover, the temporal effects of mechanical stimulation on osteogenesis and mineralization have been revealed. The kinetics of osteogenesis and mineralization depended on the temporal schedule of the application of the magnetic field, which suggested the possibility to remotely control bone growth in the magnetic bioreactor.

Works described above demonstrate that targeting magneto-mechanical activation of cell signaling pathways especially mechanical transduction is capable of augmenting the mechanical strength and reducing the in vitro preparation time of bone tissue engineered constructs. According to their distinct advantages of 'action at a distance' and 'precision afford' which have been discussed detailed in the previous review[20], magneto-mechanical actuation has been proposed for pre-conditioning of osteoblast and stem cell-seeded constructs, ultimately for the production of tissue engineered bone with enhanced differentiation, mineralization and mechanical properties.

6. IOPNs-Based Composite Bone Scaffolds

Since IONPs contribute so much in bone regeneration, bone scaffolds combine with IONPs are better choices for bone healing, especially when the therapy process accompanies the external magnetic stimulations. The magnetic scaffold is able, via magnetic driving, to attract and take up in vivo growth factors, stem cells or other bio-agents bound to magnetic IONPs, therefore can promote bone repair and regeneration. More and more researchers have been paying attention to incorporate IONPs into bone scaffolds. And the beneficial effect of magnetic scaffolds on the improvement of cell proliferation and newly formed bone tissue growth has been well documented. The scaffolds have a wide range of components,[88] including biomacromolecules (e.g., collagen,[39] silk fibroin,[35] chitosan),[89] synthetic polymers (e.g., PLA,[32] PLGA,[90] PCL,[37,91] polyethylene glycol),[92] inorganic materials (e. g. bioactive glass/glass ceramic,[93], hydroxyapatite),[39,89] and the complexes of components mentioned above. Example studies of magnetic scaffolds for bone tissue engineering and bone regeneration were summarized here.

Two strategies were considered to prepare magnetic scaffolds, which is illustrated in Figure 6. One is directly magnetizing the traditional bone scaffolds by simple physical adsorption. For example, Bock et al. developed a simple and inexpensive technique involving dip-coating of the scaffolds in aqueous ferrofluids containing IONPs coated with various biopolymers, which was able to transform commercial scaffolds made of hydroxyapatite and collagen into magnetic scaffolds.[94] He et al. incorporated superparamagnetic IONPs into HA through capillary force. The magnetic hydroxyapatite scaffolds could significantly improve cell adhesion and proliferation, and accelerate bone tissue regeneration in a beagle dog experimental model of femur transverse defect.[95]

Another route is blending IONPs with other components during the preparation of scaffolds. A few examples were reported in the following recent studies. Nanocomposite films containing Poly (3-hydroxybutyrate) and Fe_3O_4 nanoparticles were produced by moulding/particulate leaching technique.[38] The introduction of IONPs positively increased the crystallinity and protein absorption of the composite scaffolds which has been suggested to be useful in bone regeneration and was confirmed by the favorable cell attachment and proliferation of MG-63 cells. The fabrication of porous magnetic chitosan/polyethylene glycol/nano-hydroxyapatite/Fe_3O_4 nanocomposites via solvent evaporation[92] and the fabrication of magnetic silk fibroin/chitosan/Fe_3O_4 scaffolds via freeze-casting method[89] have been reported, and both of the prepared composites could be used for bone tissue engineering. In addition, magnetic Fe_2O_3/n-HA/PLLA composite scaffolds were prepared using low-temperature rapid prototyping and could induce the osteogenic differentiation of BMSCs.[32] In another research, IONPs have been also incorporated into self-setting calcium phosphate cements (CPCs).[96] The incorporation of IONPs resulted in a striking increase in cell adhesion and cell spreading area. Moreover, osteogenic differentiation which was determined by alkaline phosphatase activity was also substantially stimulated by the incorporation of IONPs.

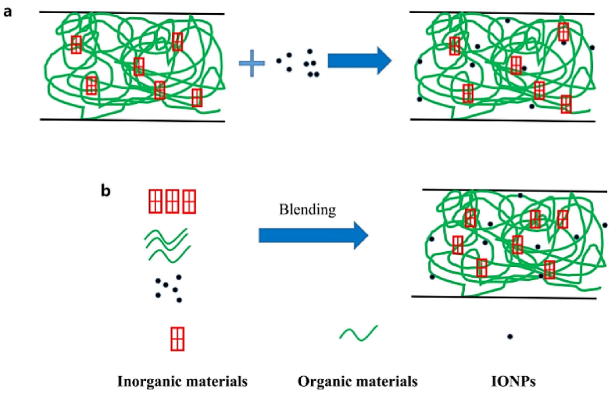

Figure 6. Two strategies to prepare magnetic scaffolds. a) Direct magnetization of the prefabricated scaffolds by techniques such as simple immersing and capillary adsorption. b) Blending IONPs with other components during the preparation of scaffolds. Almost all techniques for the preparation of composite scaffolds can be employed. Composites do not always contain both organic and inorganic materials. Only one substance, whether organic or inorganic materials, or more than two substances, are available.

As described above, different techniques were employed to prepare magnetic composite bone scaffolds. Besides the above techniques, electrospinning has a number of key advantages in the preparation of composite scaffolds especially containing polymer matrixes. The incorporation of IONPs within electrospun fibers is feasible and simple, and it has become one of the preferred jetting methodologies for the fabrication of magnetic tissue engineering scaffolds. We have prepared a nanofibrous composite scaffold composed of γ-Fe_2O_3, HA nanoparticles and poly lactide acid (PLA) using electrospinning technique. Under the applied static magnetic field of 0.9–1.0 mT, the composite film significantly enhanced the proliferation, differentiation and ECM secretion of MC3T3-E1 cells.[97] Furthermore, the scaffold accelerated new bone tissue formation and remodeling in the rabbit model of lumbar transverse defects under the static magnetic field.[33] Several aspects might be associated with the osteogenic effect of the nanofibrous composite scaffold. First is the high similarity of the scaffold to natural extra cellular matrix in the nanofibrous structure. Secondly, the incorporation of IONPs into nanofibers can give scaffolds novel function of responding the applied magnetic field. The produced magnetic forces might continually stimulate osteoblasts proliferation and secretion of new extracellular matrices. Thirdly, the applied magnetic field might be helpful to the degradation of the scaffold and thus encourage the formation of new bone.

In a more recent study, magnetic nanofibrous scaffolds of poly (caprolactone) (PCL) incorporated with IONPs were produced by Singh et al., and their effects on physic-chemical, mechanical and biological properties were extensively addressed to find efficacy for bone regeneration purpose.[37] Compared to pure PCL, osteoblastic cells favored the IONPs-incorporated nanofibers with significantly improved initial cell adhesion and subsequent penetration through the nanofibers, increased alkaline phosphatase activity and expression of genes associated with bone (collagen I, osteopontin and bone sialoprotein). What's more, IONPs-incorporated nanofibers could enhance the bone regeneration in the radial segmental defects. More studies have been described in a recent review by Mortimer and Wright, which focused on the fabrication of iron oxide nanoparticle-nanofiber composite scaffolds using electrospinning.[98]

In addition, 3D-pringting technique has been also employed to fabricate magnetic Fe_3O_4/MBG/PCL composite scaffolds.[99] The incorporation of magnetic Fe_3O_4 nanoparticles did not influence scaffolds' apatite mineralization ability but significantly stimulate proliferation, ALP activity, osteogenesis-related gene expression and extra-cellular matrix mineralization of hBMSCs.

The osteogenesis efficacy of magnetic composite scaffolds might be related to the preparation methods. Two kinds of hydroxyapatite/collagen (70/30 wt %) magnetic scaffolds were prepared with two different techniques: direct nucleation of biomimetic hydroxapatite and IONPs on self-assembling collagen fibers and scaffold impregnation in ferro-fluid solution. Implantation of magnetic scaffold prepared by the former self-assembling method showed a significantly higher bone healing rate in the rabbit models of distal femoral epiphysis and tibial mid-diaphysis defects.[39]

Some researchers have made efforts to find out the underlying mechanism by which the incorporation of IONPs would enhance both in vitro and in vivo osteogenic effects. For example, in a study, magnetic hydroxyapatite scaffolds (MHA, Fe_3O_4 nanoparticles-infiltrated HA ceramics) altered the composition of protein coronas on the scaffolds and ultimately contributed to an increased concentration of proteins related to calcium ions, G-protein coupled receptors (GPCRs), and MAPK/ERK cascades as compared with pristine HA scaffolds. The enriched functional proteins on MHA samples could efficiently activate of the MAPK/ERK signaling pathway, resulting in increased MC3T3-E1 cell proliferation.[36]

The combined effects of the external field with magnetic PCL/Fe_3O_4 nanoparticles composite scaffold on the osteoblastic functions and bone formation have also been investigated.[100] The static magnetic field synergized with the magnetic scaffolds in the osteoblastic differentiation of primary mouse calvarium osteoblasts, including the expression of bone-associated genes (Runx2 and Osterix) and ALP activity. The synergism was demonstrated in the activation of integrin signaling pathways, such as focal adhesion kinase, paxillin, RhoA, mitogen-activated protein kinase, and nuclear factor-κβ, as well as in the up-regulation of bone morphogenetic protein-2 and phosphorylation of Smad1/5/8. Moreover, the stimulated osteoblasts promoted the angiogenic responses of endothelial cells, including the expression of vascular endothelial growth factor and angiogenin-1 genes and the formation of capillary tubes.

We have fabricated the macroscopic film of IONPs by layer-by-layer (LBL) assembly on PLA scaffold, and studied the effect of the assembled IONPs film on BMCs.[34] The results showed that the LBL-assembled film could promote the growth and osteogenic differentiation of cells, especially combined with the external magnetic field. This effect stems from many aspects, of which it is worthy noted that the local magnetic ordering could be considered to account for the promotion of cellular growth. The magnetization might result in the ordered arrangement of magnetic moments to reduce the systematic energy. After the removal of external magnetic field, the thermal energy was incapable of disorganizing the magnetic moments thoroughly. Thus the remnant magnetic moments of long-range order can affect the cellular growth. We believe this novel interface between scaffold and stem cells will boost the development of next-generation scaffolds and their application in tissue engineering, bone repair and regenerative medicine.

We have also fabricated a magnetic hydrogel by assembling the IONPs in monomers solution before gelation in the presence of a magnetostatic field[101] or AMF.[102] The IONPs inside the hydrogel are anisotropically aligned. Due to the anisotropic alignment of IONPs, the thermogenesis of the hydrogel was more than that of hydrogel with disorganized IONPs and can be regulated by altering the direction of the external field with respect to the assembled chains. Doxorubicin hydrochloride (Dox) was loaded in the hydrogel, and the release of Dox can also be controlled by changing the direction of AMF. The aligned magnetic hydrogel would be designed as a

good carrier for controlled release of drugs and bone tissue repair.

Although increasing experiment data point out the clear role of IONPs stimulation on the bone cell viability and differentiation, in particular, the applied of an external magnetic field enables synergic enhanced stimulation of bone forming cells. Comprehensive investigations are necessary to understand the related mechanisms involved in the interactions between IONPs and the composite scaffolds, magnetic scaffolds and magnetic field, and the most important, the interaction of magnetic scaffolds and magnetic field with the bone cells.

7. Summary and Outlook

Employment of IONPs in bone regeneration opens a new avenue for treatment of bone defects. Many aspects of IONPs contribute to their application. The magnetic aspect of IONPs deserves special attention. Application of magnetic components has been mainly associated with the use of magnetic field, which can provide remote control of drug release and biomolecule activation and, thus, results in the biological response including cell differentiation, tissue growth and organ reconstruction. Therefore, it is very helpful to develop a suitable magnetic field for a specific application and utilize the interaction between IOPNs and magnetic field for safely and conveniently regulation the above biochemical processes. Most of the studies described in this paper are to directly use magnets or existing magnetic field generation system. Development of the magnetic-based bioreactors will promote the biomedical application of IONPs and the emergence of new IONPs-based technologies.

Moreover, it is essential to obtain better understanding of underlying mechanisms of IONPs-induced bio-effects. This is the basis for the biomedical applications of IONPs and advancement of bone remodeling. Although some analyses and experiment verifications on related mechanisms have been provided, all of them are still in the preliminary stage. A comprehensive understanding of these mechanisms can broaden the knowledge of current applications, help in obtaining more functional bone regeneration, and pave the way to translate the in vitro and in vivo work into further orthopedic clinical studies. In this regard, more research must be conducted in this direction.

Finally, despite definite advantages and huge therapeutic capacity of IONPs-based materials, clinical applications must be carried out cautiously with respect to possible adverse effects of IONPs on organism. The sizes of IONPs range from a few nanometers up to tens of nanometers. This place IONPs at dimensions smaller than or comparable to those of a cell (10–100 μm), a virus (20–450 nm), a protein (5–50 nm) or a gene (2 nm wide and 10–100 nm long), so IOPNs can 'get close' to a biological entity. On the one hand this is the basis for the application of nanoparticles, and on the other hand, there may be a risk of unknown to us. The effects of IONPs on biological substances should be thoroughly investigated. Some omics studies such as metabolomics, proteomics, and genomics may also be necessary. In addition, despite the existence of physiological iron metabolic pathways, the tissue distribution and the excretion of additional iron oxide nanoparticles still deserves our attention.

Acknowledgements

The work was supported by grants from the National Basic Research Program of China (No. 2013CB934400), the National Key Research and Development Program of China (No. 2017YFA0104301), the Natural Science Foundation of Jiangsu Province (No. BK20130608), and the Fundamental Research Funds for the Central Universities (No. 2242017 K40232).

Conflict of Interest

The authors declare no conflict of interest.

Keywords: iron oxide · magnetic properties · nanotechnology · osteogenic differentiation · regenerative medicine

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Manuscript received: November 30, 2017
Version of record online: April 14, 2018