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Engineered Magnetic Nanocomposites to Modulate Cellular Function

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Magnetic nanoparticles (MNPs) have various applications in biomedicine, including imaging, drug delivery and release, genetic modification, cell guidance, and patterning. By combining MNPs with polymers, magnetic nanocomposites (MNCs) with diverse morphologies (core–shell particles, matrix-dispersed particles, microspheres, etc.) can be generated. These MNCs retain the ability of MNPs to be controlled remotely using external magnetic fields. While the effects of these biomaterials on the cell biology are still poorly understood, such information can help the biophysical modulation of various cellular functions, including proliferation, adhesion, and differentiation. After recalling the basic properties of MNPs and polymers, and describing their coassembly into nanocomposites, this review focuses on how polymeric MNCs can be used in several ways to affect cell behavior. A special emphasis is given to 3D cell culture models and transplantable grafts, which are used for regenerative medicine, underlining the impact of MNCs in regulating stem cell differentiation and engineering living tissues. Recent advances in the use of MNCs for tissue regeneration are critically discussed, particularly with regard to their prospective involvement in human therapy and in the construction of advanced functional materials such as magnetically operated biomedical robots.

1. Introduction

The functional modulation of cells and tissues has been traditionally carried out by tethered invasive systems that apply physical forces to the intended sites of action (e.g., surgery) and by remote molecular stimulation via biochemical conditioning, which allows for the biomanipulation of protein functions, intracellular signaling pathways, and animal behaviors.[1] For instance, in chemogenetics, selective pharmacological control over diverse cellular functions can be mediated by small molecules, which interact with the engineered receptors that affect the cell-signaling processes.[2,3] However, the perturbation of cellular functions through genetic and pharmacological techniques only allows for basic on/off switching.

In contrast, a second generation of remote stimulation techniques based on physical tools has enabled biological targets to be precisely controlled, both spatially and temporally.[1] Consequently, a wide plethora of approaches has been developed in recent years, including the use of electric fields, light irradiation, heating, ultrasound, pH variations, and magnetism.[1,4–8] Biophysical cell stimulation fosters specific cell behaviors through mediating and/or converting various forms of energy into physicochemical cues; therefore, it has dramatically impacted fundamental, methodological, and therapeutic sciences.[9] For example, a revolutionary dissection of signaling networks and neural circuits has been achieved as a result of the discovery and implementation of optogenetic regulation concepts.[10]

More recently, certain categories of nanomaterials, termed "smart nanomaterials," have been recognized as useful transducing tools; their ability to change structural and functional properties in response to specific external stimuli allows them to mediate the remote manipulation of cells.[1] In particular, methods that exploit the interaction of living systems with magnetic fields (MFs) and/or materials endowed with magnetic responsiveness have emerged.[11–13] For instance, magnetic nanocomposites (MNCs) are composite materials with magnetic behavior[14,15] that arise from the hybrid combination of magnetic nanoparticles (MNPs) and polymers. By definition, organic-inorganic MNCs generally comprise MNPs embedded in a nonmagnetic or magnetic matrix.[16] Recent developments

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in materials science have rendered MNPs easy to synthesize and accommodate into these composite platforms, resulting in the generation of multifunctional MNCs that have brought tremendous advances in biomedicine, particularly in relation to therapeutic delivery and biological system engineering.[14–16] Furthermore, MNCs have been proposed as a novel biomaterial for cell culture and tissue maturation in vitro due to their ability to trigger a biological response to external stimuli carried via an external static magnetic field (SMF) or alternating magnetic field (AMF).[14,16] These stimuli are transformed into physicochemical factors that can alter biochemical processing, nutrient metabolism, molecular signaling, and gene expression in the cells.[14,15]

Here, we present a comprehensive overview of various types of MNPs, polymers, and other additive materials, as well as several combinations of the above, in use to generate MNCs for biological fundamental investigations and applications. In the following sections, the ability of these constructs to interact with cells and trigger effects at a cellular and molecular level will be discussed, with the aim of inspiring scientists to develop novel biomedical technologies based on the MNC concept. To such a purpose, we will illustrate the effects of polymeric MNCs on various aspects of the cell behavior that include: differentiation, proliferation, adhesion, migration, phenotype polarization, death, and metabolism. In particular, this review will focus on MNCs which have proved to be advantageous in the generation of 3D cell culture models and in vitro maturation of grafts for tissue regeneration and reconstruction, including examples that are close to being used in clinical practice. Finally, the design of advanced MNCs and magnetic control paradigms will be presented, and the methods for spatial cell guidance and patterning, along with their implementation into small scale robotic structures under noncontact magnetic control, will be described.

2. Magnetic Nanocomposites

A nanocomposite (NC) is an inhomogeneous solid material in which at least one of its phases measures less than 100 nm in one, two, or three dimensions, or in which the interphasic distance repeats in the nano-scale range.[18] Examples of 1D nanoscaled materials are very thin layers and surface coatings. Nanotubes and nanowires are considered 2D nanoscaled materials.[19,20] Particulate materials with all three external dimensions on the nanoscale are called nanoparticles (NPs).[18] The properties of an NC arise as a result of a combination of the typical features of matrix materials and the nanosized fillers. While a vast variety of host materials are used in the generation of nanohybrids (including silica, organic polymers, or even liquid media), NPs primarily emerge through the functions provided by the added nanofillers.

NPs display a high surface-to-volume ratio and high total interfacial area, which, together with their reduced size, allow them to interact optimally with the molecular and supramolecular entities that are present in biological environments. For instance, a NP's size is comparable to that of biomolecules such as proteins (1–20 nm), cell membranes (≈6–10 nm), DNA (≈2 nm in diameter), hemoglobin (≈5 nm), and viruses (≈20 nm). Thus, NPs diffuse across cell membranes more easily, and are more suitable for intravenous administration, than larger objects such as micro-particles. Furthermore, the tunability of NPs' properties renders them superior in terms of their potential in controlled drug release and site-specific drug targeting.[21] As a result, techniques for bio-sensing, molecular detection, gene delivery, DNA transfection, viral transduction and the remote guidance of cells can be mediated by nano-objects. When they are embedded into multiphasic systems, NPs composed of carbon, metal, ceramic, polymers, semiconductors, or lipids can provide the hosting material with additional functionalities, which derive from their own key-features (e.g., mechanical resistance, elasticity, degradability, electrical conductivity, etc.). However, when NPs are integrated into a matrix, they tend to aggregate due to a reduction in the energy that is associated with their high surface area-to-volume ratio. Given that this phenomenon offsets any benefit derived from their small dimensions, current strategies in NC preparation aim to create homogeneous dispersions of isolated NPs.[22]

MNPs have been employed in many biomedical applications: gene and drug delivery; cell separation and labelling; imaging and remote guidance of cells in vivo; hyperthermia treatments; cell patterning in the generation of 3D tissue constructs; and the activation of intracellular pathways.[23,24] MNPs consist of metal oxides, ferrites, metallic or bimetallic NPs, and superparamagnetic iron oxide NPs (SPIONs). Given that MNPs tend to agglomerate when they are dispersed into composites, chemical strategies to avoid particle aggregation have been developed. These strategies rely on stabilizing the naked core by coating it with an inorganic layer (e.g., carbon or silica) or by functionalizing or coating it with organic species (i.e., polymers or surfactants). Such treatments result in a more effective incorporation of the nanoparticulate into matrices of various natures.[25]

Most relevant for this review, MNPs serve as nanofillers to generate MNCs.[14,15] The resulting MNCs have been useful in several fields of material and environmental sciences, such as catalysis, information technology, environmental remediation (*e.g.*, for the removal of heavy metals, toxic effluents and oils), telecommunication, etc.[16,26] More intriguingly, MNCs have also been used in biomedicine; in particular, they have shown promise in the field of cancer therapy and diagnosis, as they can be used via targeted drug delivery and magnetic resonance imaging (MRI).[27]

MNCs can be categorized as self-assembled, core–shell, or organic-inorganic, and they display various morphological configurations (as is shown in **Figure 1**). The first category is 2D and 3D macrostructured superlattices, which are generated by the self-assembly of NPs. Here, the NPs create superstructures that are characterized by a translational and orientational order with a crystallographic alignment.[28,29] For instance, colloidal crystals or quasi crystals from MNPs have been reported.[30] The second category is based on the concept of core–shell inorganic NCs, which involves combining two nanoscaled entities into a single hybrid particle. Since these particles are typically composed of shells (outer layers) and cores (inner materials), they exhibit better functional properties and performance due to the interactions between the constituent phases.[31] For example, silica has been widely employed to coat and encapsulate MNPs in view of diverse environmental and biological uses.[32] In addition to its high hydrophilicity, biocompatibility, physical

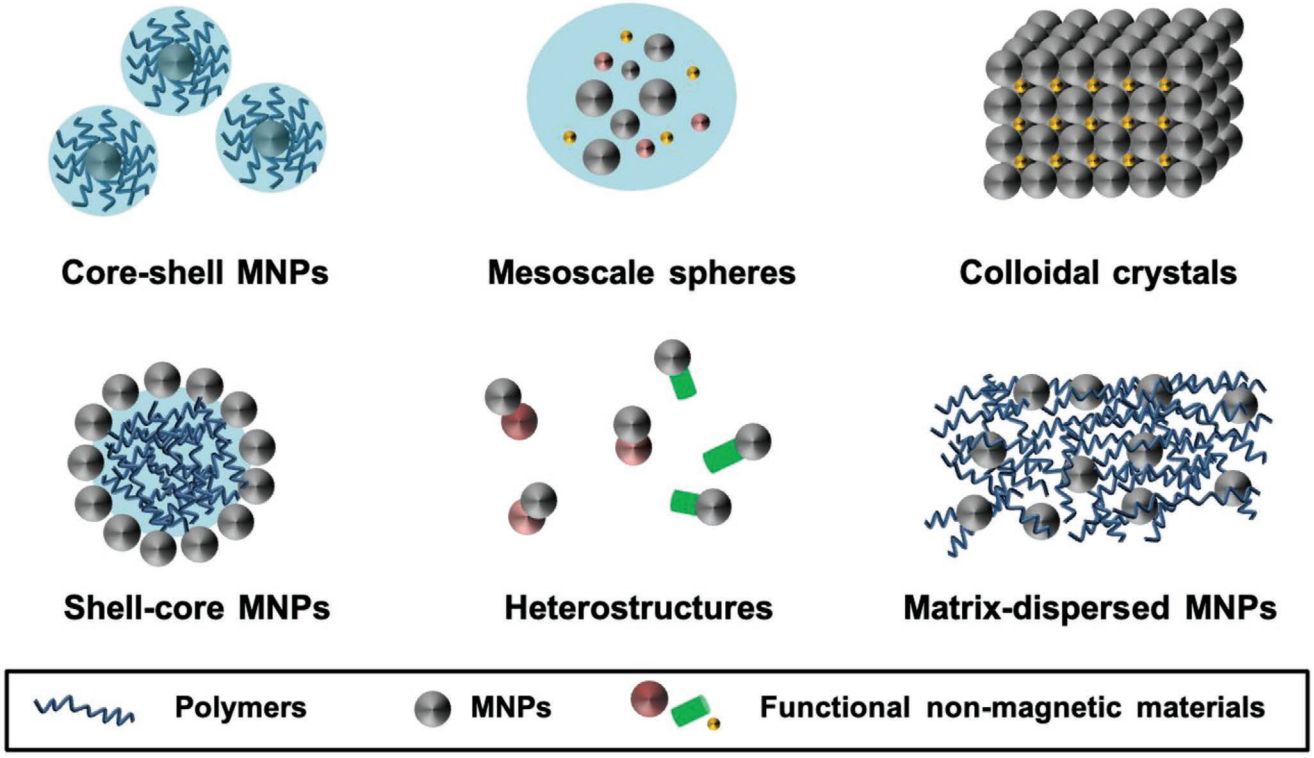

Figure 1. Various morphologies of MNCs. Core–shell MNPs are protected by an organic coating (e.g., polymers, ligands), whereas MNPs constitute the external layer in shell–core systems. In mesoscale spheres, MNPs are dispersed in mesoscale spherical assemblies. Heterostructures (Janus type) are bimodular materials composed of one magnetic and one nonmagnetic functionality. MNPs can be dispersed in polymeric matrices or organized in ordered crystalline configurations.

resistance against degradation, tunable porosity, and large surface area of its mesoporous architecture, silica is also highly versatile for functionalization due to the presence of silanol surface groups, which can be easily derivatized with a variety of chemical functionalities.[32,33] The third category is organic-inorganic MNCs, which typically comprise NPs that have been finely dispersed in a polymeric environment. These NPs display a combination of unique properties that derive from the organic and inorganic counterparts. Organic materials composited with nano-sized fillers exhibit rheological behavior, which is strongly dominated by the NP surface area and the resulting huge polymer-filler interfacial layer, rendering the properties highly tunable.[27,34] Thanks to their dual nature, these hybrid organic-inorganic MNCs have gained much interest over the last decade, and they have been utilized to make great advancements in the domains of biomedicine, electronics, microoptics, and energy conversion or storage. Moreover, a variety of protocols have been proposed for their preparation, including in situ and ex situ synthesis, melt blending, microwave reflux, coprecipitation, plasma polymerization, and ceramic-glass processing techniques.[27]

2.1. Main Functional Components of MNCs

MNPs and polymers are the main components that are required for the preparation of MNCs. In this section, the main properties of MNPs and polymers will be highlighted in order to clarify how they can be combined and synergically work together in hybrid composite systems. First, the key properties of MNPs, which can mediate the exceptional ability to remotely control the whole composite material due to their magnetic responsiveness, will be described. Then, the main types of polymers that are used as protective coatings or bulk embedding materials within the MNCs will also be presented. This section will facilitate an understanding of the functional behavior of MNCs, and their ability to affect the biological processes of living systems, which will be reported in the subsequent sections.

MNCs can also contain other components that provide structural reinforcement or other functions (e.g., electrical conductivity, enhanced elasticity, mechanical resistance, etc.).[14–16,26] The nature of these additional components strongly depends on the MNC application. This review will focus on the description of MNPs and polymers as two main functional components.

2.1.1. MNPs

MNPs are NPs endowed with a magnetic behavior.[14,15,23,24] The degree of magnetic ordering and the sample temperature are the two main factors affecting the magnetic response of MNPs to an externally applied MF. The magnetization is defined as the magnetic moment per unit volume of a particle, and mostly depends on the spin or the orbital energy possessed by the dipole. The saturation magnetization corresponds to the maximum induced magnetization upon MF application, whereas the remanent magnetization is the induced magnetization that remains even after the field is removed. MNPs can be produced

through a variety of methods (such as hydrothermal techniques, coprecipitation, sol–gel processing, surfactant-assisted synthesis, microemulsion techniques, and solution combustion) that aim to control the morphology, stability and dispersion of formulations. These methods can be used to create different types of MNPs, including: polymer or plastic magnets, dilute magnetic semiconductors, ferrites, metal, and metal oxide nanoparticles.[27]

Polymer or plastic magnets are nonmetallic magnets composed of organic polymers, and they are particularly useful in engineering computer hardware and medical devices.[35] For instance, when poly(1,3,5-triaminobenzene) is oxidized with iodine, it presents a ferromagnetic phase up to 400 °C.[36] Magnetic polymers that function at room temperature have also been developed.[37]

Dilute magnetic semiconductors are materials containing isolated magnetic ions that have been dispersed in a semiconducting lattice. They mainly consist of Sn, Zn, and Ti oxides, or mixed oxides that have been doped with several transition metals or rare earth metals. Dilute magnetic semiconductors show promise in spintronic device applications.[38–41]

Ferrites are ferromagnetic materials that, according to their resistance to being demagnetized (i.e., magnetic coercivity), can be divided into hard and soft ferrites characterized by a high and low coercivity, respectively.[42] Ferrites display three different structural symmetries (garnet, hexagonal, and cubic or spinel ferrites), and have been applied in magnetic drug delivery, MRI, catalysis, sensors, permanent magnets, and the preparation of ferrofluids.

Transition metals such as iron (Fe), Ni, and Co display ferromagnetic behavior at low and room temperatures, while they exhibit paramagnetism at high temperatures. NPs composed of metal oxides have also been widely studied, including α-Fe_2O_3 (hematite), γ-Fe_2O_3 (maghemite), Fe_3O_4 (magnetite), hexagonal ($MFe_{12}O_{19}$), garnet ($M_3Fe_5O_{12}$), and spinel (MFe_2O_4), in which "M" represents one or more bivalent transition metal (i.e., Fe, Co, Ni, Ba, Mn, Sr, Zn, or Cu).[43] In particular, compounds based on iron oxides, hydroxides, or oxide-hydroxides, which are grouped under the name of iron oxide NPs (IONPs), have emerged for biomedical and industrial use.[44]

Hematite, magnetite, and maghemite are the most relevant typologies of IONPs.[45] The diameter and magnetic domains of ferromagnetic particles have been extensively discussed, as well as their biomedical versatility.[46,47] Examples of applications include: MRI; sensing; drug delivery; cell labelling and sorting; MF-assisted cancer therapy (hyperthermia); the removal of heavy metal ions; the catalysis of NH_3 synthesis; large-scale butadiene synthesis; Fisher-Tropsch hydrocarbons synthesis; the oxidation of alcohols; and other reactions. Below a critical particle size, ferromagnetic NPs consist of a single magnetic domain, presenting a uniform magnetization across any field. Furthermore, they display a magnetization behavior that matches that of atomic paramagnets when they are above a certain temperature by involving large susceptibilities (i.e., superparamagnetism). These superparamagnetic IONPs (SPIONs) exhibit a unique magnetism, which arises from a combination of their crystal structure, atomic composition, and size effects. As per definition, the hysteresis for superparamagnetic materials is actually null. However, ensembles of SPIONs produce a hysteresis loop with a minimal remanence and coercivity when larger particles that derive from aggregation are present.[48] SPIONs are regarded as chemically inert materials, and they are great tools for imaging, targeting, drug delivery and biosensing as a result of their low toxicity, potent magnetic and catalytic activity, and superior role in multifunctional modalities.[49,50]

The movements of particles that are endowed with mass and electric charges (such as electrons, protons, anions and cations) give rise to magnetic effects. Besides their electrical, structural, gas sensing, and optical properties, MNPs demonstrate magnetic properties based on their magnetic susceptibility (χ), which corresponds to the ratio of the induced magnetization (M) to the applied magnetic field (MF).[51] Important parameters to characterize the magnetic behavior of MNPs are: the saturation magnetization, remanence and coercivity, the magnetocrystalline anisotropy constant, and the mechanism of magnetic relaxation.[24,51] All of these properties depend on the specific magnetic material, the NPs' synthesis method and coating, and the procedure for sample preparation before measurement. Some of the notable features found in MNPs include: superparamagnetism; magnetic anisotropy affected by shape and inherent crystalline structure; magnetic saturation, and loss of hysteresis (irreversibility) occurring at high fields; and shifted loops after field cooling. These features derive from the fact that the magnetic behavior of individual NPs is dominated by physical factors like narrow and finite-size effects, and surface effects.[45–51] Moreover, in addition to the controllable magnetic activation and high magnetization of MNPs, they display other unique advantages: they have effective surface areas, low sedimentation rates, and high tissular diffusion; and they are colloidally stable under physiological environmental conditions (aqueous media, pH 7, 37 °C, etc.).[14,15] To further adapt the MNPs for biomedical use, the charge and surface chemistry can be modulated, biocompatible layers can be used for encapsulation, and systems for drug entrapment can be established.[52]

2.1.2. Polymers

The incorporation of hard inorganic nanomaterials into a soft organic matrix enhances the chemical, physical and biological properties of several polymer systems such as hydrogels.[53] Polymers have been extensively used to engineer a multitude of materials with controllable thermal, mechanical, and electroactive functions.[27] Polymers of either synthetic or natural origin can be processed to obtain diverse matrix structures.[54–57]

Natural polymers derive from the metabolic activity or other physiological processes of living beings; therefore, they are highly biocompatible and biodegradable. Consequently, they are often used to replicate living microenvironments since there is a high degree of similarity between them and the natural extracellular matrix (ECM). Cell recognition and adhesion sites are also present.[55,58] However, the characteristics and the biological functionality of natural polymers strongly depend on the specific source and extraction process.[56,58] The variability and the difficult standardization of their manufacturing process can be overcome by using polymers of synthetic origin, whose mechanical and physicochemical features can be precisely

tailored during preparation in order to match those found in biological tissues.[59,60]

The most widely used synthetic polymers for NCs for biomedical applications include poly(α-ester)s, poly(esteramide)s, polyurethanes, polyanhydrides, polyacetals, polyphosphazenes, and pseudo poly(aminoacids).[57,61] For instance, porous, interwoven, rigid materials that support cell growth can be formed from the nanofibers of poly(methyl methacrylate) (PMMA), polyethylene terephthalate (PET), polyhydroxyl acids (PLA, PGA, PLGA), polyhydroxybutyrates (PHB), polyhydroxylketones (PHK), poly-ε-caprolactone (PCL), polyvinyl alcohol (PVA), and polyhydroxybutyrate-*co*-hydroxyvalerate (PHVB).[57,61]

Polymers have been widely manipulated to obtain nanofibers with a bio-inspired morphology in order to replicate the ECM structure in scaffolds so that they can host and develop cell cultures.[62] In fact, submicron polymer fibers can generate a mesh with high porosity and pore interconnectivity, which facilitates cell spreading and networking, as well as the efficient transfer of nutrients and waste products. Moreover, the huge surface-to-volume ratio of nanofibers allows proteins to be binded, which, in turn, favors cell adhesion. While generating biomimetic 3D architectures with the tailored surface properties at the microscale, polymeric nanofibers also impart mechanical strength to the macrostructure. For these reasons, in biomedicine they have been used to prepare scaffolds for tissue regeneration, wound dressing, and drug delivery. Although polymeric nanofibers can be obtained by various methods (such as enzymatic digestion, phase separation, and self-assembly), electrospinning has emerged as a simple, versatile and effective technique to produce nanofibrous layers.[63] Electrospinning exploits the interaction of a charged polymeric liquid with a strong electric field and, since it only requires a high voltage supply to convert a polymeric solution to solid nanofibers, it has been applied in the nanofabrication of hundreds of polymers to date, and consistently used on the industrial scale.[62]

2.2. Magnetism of MNCs

The discovery of novel and more sophisticated 3D geometries in nanostructured magnetic materials has greatly broadened the horizons of nanomagnetism.[64] Nanomagnetism aims to unravel the physical processes that occur at the nanoscale and to determine which and how parameters affect the interpretation of the measured magnetic properties.[64,65] Each MNC system requires a simultaneous assessment of the effects that derive from its structure, composition, and morphology. Such an evaluation requires the system to be modeled through a detailed magnetic and morphological characterization.

The strength of the interaction between the NPs that are composed of different transition metals, their degree of dispersion and aggregation, and the effect of the matrix surface on their magnetism can affect the overall behavior of the MNC.[66,67] Since the morphological features of NPs play a crucial role in determining differences in the magnetic properties of MNCs, their coupling to the matrix (independent vs bounded), their mutual interactions (isolated vs aggregated), and the nature of the colloidal dispersion (mono- vs poly-dispersed) are all relevant aspects to be considered when designing MNCs. When NPs are present in low concentrations, the resulting magnetism can be described in terms of weakly interacting or non-interacting NPs. MNCs with higher concentrations of MNPs display anisotropic behavior, which arises from the generation of mesoscopic clusters of MNPs, which, in turn, behave as single magnetic units.

Manufacturing methods can also affect the magnetic strengths of MNCs. One approach consists of first preparing the NPs in the form of a nanopowder, which is subsequently dispersed in a fluid matrix via mixing and stirring. The matrix then eventually undergoes solidification.[68–70] Loading MNPs into a polymer fluid resin in this manner is a conventional approach to produce polymeric MNCs. An alternative method relies on the simultaneous generation and coassembly of the NPs and the host matrix. For instance, ceramic NCs can be generated in this way by thermally treating zeolite precursors that have been loaded with transition metals.[71]

As a result of their composition and magnetic behavior, MNCs are capable of interacting with living systems such as cells and tissues.[14–17] In the next section, the underlying mechanisms behind this interaction will be reviewed in detail.

3. Interacting with Cells through Magnetism

A variety of effects have been documented for cells and tissues that have been exposed to MNPs, AMFs, SMFs, or a combination of the above.[72–78] In this section, we will briefly introduce the effects that can be induced by MNPs alone, before discussing how they can be combined with applied fields in order to modulate the biological processes that are active within the cells.

3.1. Mechanisms of MNPs–Cell Interaction

Depending on their composition, MNPs can affect a cell's homeostasis from a chemical point of view.[79,80] Upon cell internalization, certain MNPs experience transformations that often result in their progressive endosomal degradation. IONPs can be degraded to elemental iron, thus altering the intracellular iron homeostasis. However, this process is tightly regulated by proteins, such as ferritins, so that the cell metabolism can adapt to the amount of iron.[81] Moreover, the MNPs act as a single magnetic domain, providing an MF at a nanoscale level, even in the absence of an externally applied field.

The interaction of MNPs with an externally applied MF and with other MNPs present in the microenvironment results in energy changes within the system, and these changes redefine their expected biological activity.[52] Combined MNPs and MFs can affect the cellular biochemical processes, and they can be used to interact with cells via three different approaches (**Figure 2**).[82–84] The first is a spin-dependent mechanism that has been extensively studied and well-established. This technique is at the base of MRI development, has been implemented in medical spintronic technology, and most widely applied to cell tracking via imaging and the assessment of contrast variations.[85,86] The second mechanism is magnetic hyperthermia, which aims to locally deliver thermic energy by

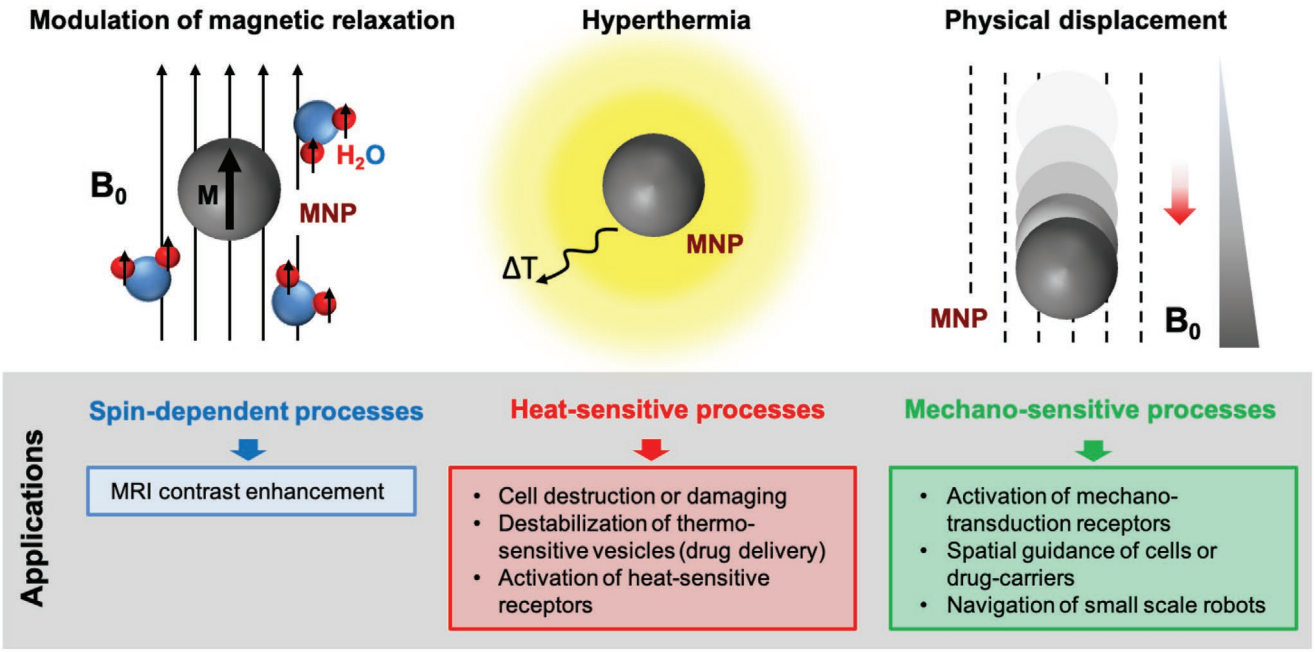

Figure 2. Interaction mechanisms between magnetism and biological systems. MNPs and MFs can interact with living systems (cells, tissues) by exploiting: i) spin-dependent processes for cell imaging, ii) hyperthermic processes that cause a local increase of temperature, and iii) mechanosensitive processes that are useful for various applications, including the direct physical stimulation and displacement of cells.

subjecting MNPs to AMFs. The heat produced can be utilized for cancer cell destruction, drug release from heat-sensitive vesicles, and heat-sensitive receptor activation.[87] Heat-induced cellular effects have been already extensively reviewed.[88] Finally, the third mechanism occurs when the acquired energy exerts magnetomechanical effects on cells when MNPs are subjected to gradient or homogeneous SMFs.[52,89] This results in a remote control strategy that is useful for magnetic targeting and mechanosensitive receptor activation.

Magnetomechanical stimulation is an important tool in medical research, as it enables to physically control cells and subcellular structures.[52,89] MNCs can be used not only to create nano-actuators that activate specific signaling cascades by interacting with the receptors and channels of the cell membrane, but also to generate motile systems for a targeted delivery of cells and substances (such as guided carriers and small scale robots) (Figure 2). As such, the following paragraphs will cover various principles and aspects for remote magnetic cell function modulation, with a focus on the magnetomechanical effects.

3.2. Magnetic Actuation of Cells and Tissue Regeneration

In biological modulation mechanisms that are grouped under the definition of "magnetic actuation," MFs can be converted into mechanical stimuli or heat, acting as a cue in regulating biological processes. This occurs because specific magnetic materials react to MF exposure by functioning as actuators (namely, effectors which apply a mechanical stimulation on cells), interacting with the cytoskeleton components,[11] or molecular receptors and ion channels that are exposed on the cell membrane.[90,91] In particular, magnetic actuation enables intracellular or cellular stimulation via four main routes: i) magnetic force, ii) magnetic torque, iii) the control of molecular aggregation, and iv) heat generation (**Figure 3**).[92]

MNPs that are surrounded by various biological polymers can serve as magnetic actuators, operating in both intracellular and extracellular compartments.[93,94] In fact, they: permit protein positioning within cells; allow the cytoskeleton to be spatially engineered; enable mastery of ion channels; and mediate membrane receptor activation.[95] For instance, high-performance magnetism-to-mechanical force transducers, called "magnetic nanotweezers" (MNTs), are composed of force-generating MNPs and field generators.[96] MNTs can deliver controlled mechanical stimulation to targeted biomolecules with diverse spatial (single molecule/cell to organisms) and temporal (microseconds to days) resolutions. SPIONs have proved to be effective not only at gathering in certain locations in vivo,[97,98] but also at clustering the cell surface receptors, applying direct actuating forces on the Integrin and Notch receptors, and even activating temperature-sensitive ion channels, like the transient receptor potential vanilloid 1 (TRPV1), a cation channel with an activation thermic threshold of 42 °C.[99–101] Similarly, both AMFs and SMFs could activate neurons that have certain temperature- and mechanically sensitive ion channels; these ion channels were functionalized via molecular fusion to ferritin.[102,103]

Magnetic actuation becomes particularly interesting when it is applied to crucial regulators in tissue homeostasis. For example, stem cells (SCs), which are essential for regeneration under both physiological and pathological conditions,[104] have become attractive tools in medicine and in engineering living tissue in vitro. SC therapy has had positive outcomes

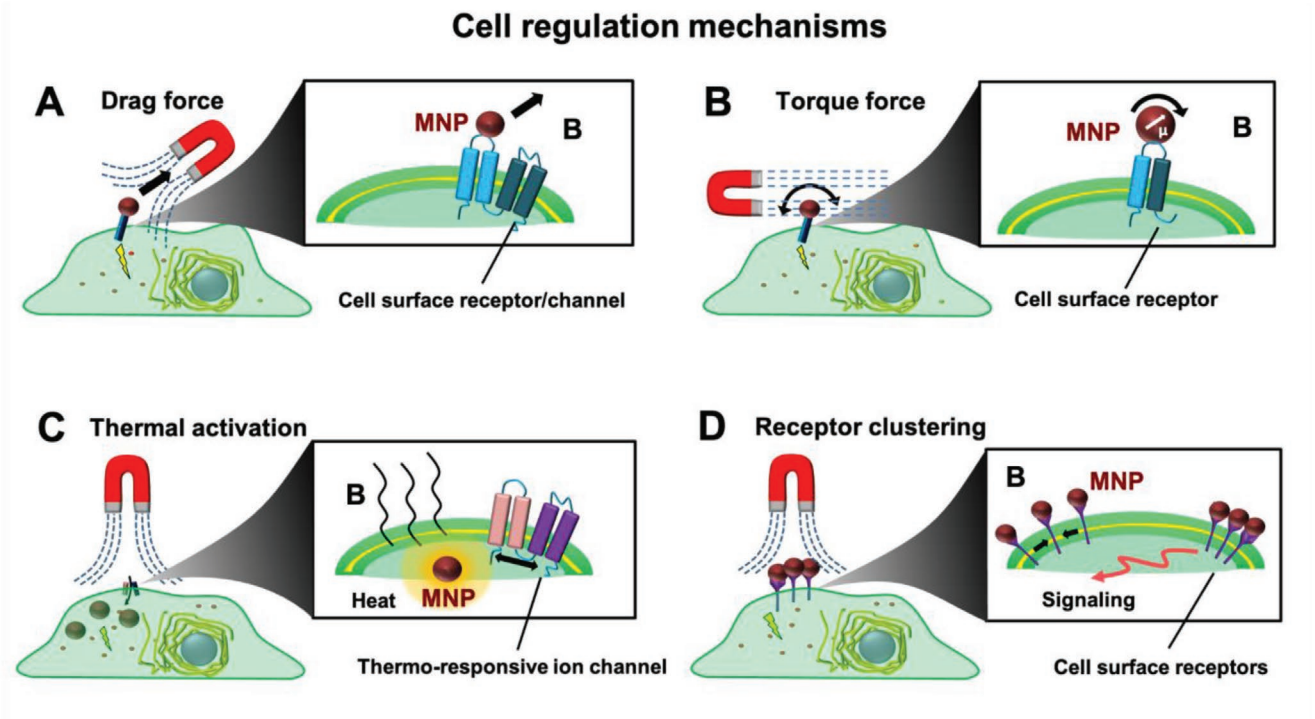

Figure 3. Modulation of biological cell behavior via magnetic actuation. Intracellular signaling can be magnetically stimulated by MNPs that operate A) drag or B) torque forces on the receptors that are present on the cell membrane, C) modulate the activity of thermosensitive channels or receptors, or D) cause receptor aggregation and start intracellular signaling.

in preclinical and clinical trials in several different fields; it shows great promise for the treatment of cerebrovascular diseases, autoimmune disorders, and tumors. Furthermore, it has potential for wound repair and in the engineering of tissues for transplantation.[105] Various types of SCs can be used for these different purposes. Adult SCs, such as adult mesenchymal stromal cells (MSCs), are currently applied to the regenerative therapy of stroke, ischemic heart disease, and tendon defects. In contrast to embryonic SCs (ESCs), they avoid ethical concerns regarding harvesting SCs from fetal tissue.[104,105] The progenitor features in MSCs are predominantly defined by the tissue of origin, namely the umbilical cord blood, bone marrow, or adipose tissue. For example, bone marrow derived MSCs (BM-MSCs) can differentiate into several nonhematopoietic cell types. These cell types play a fundamental role in tissue repair and remodelling.[104,105] whereas adipose-derived MSCs (ASCs) successfully regenerate osteogenic and chondrogenic tissues in animals.

It has been shown that SCs can assimilate IONPs, store the iron released from their degradation in endogenous ferritin, and modify their metabolism accordingly.[81] Interestingly, SCs display enhanced cell adhesion, proliferation, and viability, as well as a better differentiation potential, when they are exposed to external MFs and magnetic materials.[106,107] Their versatility renders IONPs and magnetic forces as one of the most intriguing options for the future design of integrated approaches to SC therapy. Furthermore, over the last few years, many researches have focused on the development of magnetically actuated NCs for potential use in the field of tissue engineering (TE), and there is mounting interest in the remote control of SCs and other cell types. One possible configuration for such studies would be NCs assembled from MNPs and polymers, which would result in cells being able to find suitable microenvironments for the establishment of functional niches, growth and development into mature tissue. The constructs could then be exposed to external MFs.

There is still much knowledge to be gained regarding the exact underlying mechanisms behind the augmentation of the bioactivity imparted to cell scaffolds by magnetization.[108] More precisely, much can be learned about the complex biological dynamics that affect the overall performance of cell cultures on 3D magnetic substrates; such performance depends on various cellular processes, including proliferation, adhesion, differentiation, migration, and metabolism. Furthermore, magnetism can affect these phenomena to different extents, according to the specific experimental settings. In porous MNCs with incorporated IONPs, the migration, differentiation, adhesion, and proliferation of cells increased even in the absence of MFs.[109,110]

One explanation relies on the magnetocaloric effect, which is caused by the Earth's MF. Also known as "adiabatic demagnetization," this magnetocaloric effect is a magneto-thermodynamic phenomenon in which changing MFs determine thermic variations within a suitable material. Zhao et al. incorporated nanohydroxyapatite (nHAP) and magnetite SPIONs into a chitosan/collagen organic matrix, seeded it with osteoprogenitors, and postulated that the magnetocaloric effect of the MNPs in the terrestrial MF might facilitate cell proliferation in 3D culture models for bone tissue regeneration.[110] However, the nHAP nucleation was augmented in this system, a fact that is ascribed to the presence of nanoparticulates, which indicated that IONPs

can provide a superior microenvironment for cell proliferation by affecting the mineralization processes.

In addition to the Earth's field-induced magnetocaloric effect, the magnetic actuation of MNCs for cell culture in the presence of externally applied MFs has also recently emerged. It has been proposed that such an actuation can deliver a physical stimulus to which the cell tries to adjust.[88] This causes tension in the cytoskeleton, a common phenomenon in cell proliferation and spreading,[89] which has also been correlated with SC differentiation.[88,90] The following paragraphs will cover the latest advancements in cell culture on MNCs, and present the causative mechanisms postulated to alter the cellular behavior.

4. Cell Processes Affected by Magnetism

Magnetic materials and forces can affect various biological processes in cells, including: differentiation, proliferation, adhesion, migration, phenotype polarization, death, and metabolism (**Figure 4**). As such, they can be used to remotely control living materials in biomedical applications such as tissue repair. This section will describe how MNPs and MFs can modulate different cell behaviors, and will discuss the magnetic actuation of SCs in TE applications.

4.1. Differentiation of Stem Cells

Although it has not been confirmed, it is likely that the successful outcome of SC therapy strongly depends on the use of MSCs that have been extracted from the same organ as the damaged tissue that is to be repaired.[105] Given that it is not always feasible or convenient to collect cells from these areas, scientists from all over the world have become fascinated by the idea of manipulating SC differentiation and optimizing it for specific tissue regeneration. In 1977, H. Green was the first to hint that cell differentiation could be regulated via cell–ECM adhesion mechanics, and not only by soluble growth factors.[111] In the following decades, mounting evidence demonstrated that, in addition to accelerating or enhancing the various types of biological behavior that SCs exhibit, magnetic substrates and fields can also affect SCs' fate specification process by strongly committing them toward the osteogenic lineage.[11,90,91,112] For instance, when IONPs are combined with pulsed electromagnetic fields (PEMFs), they can induce migration and osteoblastogenesis in bone marrow-derived MSCs (BM-MSCs).[97] Consequently, MNPs have gained much interest with regard to bone TE.[79] In addition, the magnetic remote control can be applied to other functional compartments of the muscolo-skeletal apparatus (muscles, cartilage, tendon, and vasculature),[113–117] and other tissue types (e.g., neural, immune

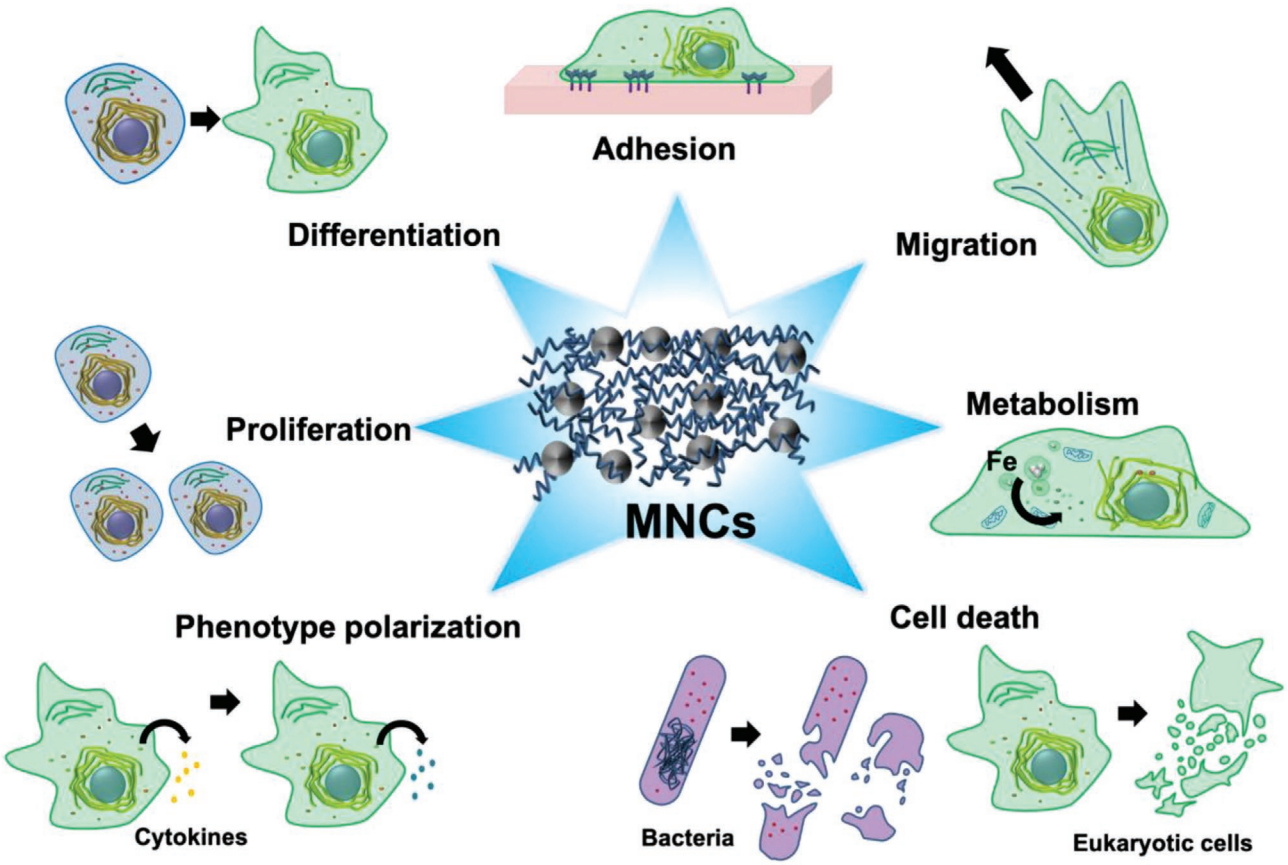

Figure 4. Cell functions modulated by MNCs and MFs. Cellular processes that can be regulated in magnetically stimulated cell cultures include: differentiation, proliferation, adhesion, migration, phenotype polarization, cell death, and metabolism.

cells).[118] The next paragraphs will discuss the use of MNCs in modulating SC differentiation for bone, tendon, and neural phenotypes.

4.1.1. Osteogenic Differentiation

Bone is a natural composite that comprises organic and inorganic materials. The main constituent of the inorganic phase is crystalline HA, while fibrous collagen is the most common organic substance. Over the last few years, various researchers have explored the use of MNCs and MFs in the development of grafts for bone regeneration and repair. Implantable magnetic scaffolds for bone tissue replacement can be obtained either by direct structural enrichment with magnetic NPs, or by seeding with magnetized progenitor cells.[12,119] Cell exposure to MFs and/or to scaffolds with intrinsic magnetic properties regulates several biological responses. Specifically, remote magnetic stimulation acts as an instructive mechanical cue that activates the mechanotransduction signaling pathways, resulting in osteogenic cell differentiation.[11,120,121] Thus far, most investigations have been conducted on osteogenic precursors (osteoblasts, preosteoblast) or stromal cells (MSCs, ASCs) of mammalian origin (human, mouse, rat), which have been cultured in an inducible cell culture medium in the presence of common biochemical differentiation factors such as dexamethasone, ascorbic acid, and beta-glycerophosphate.[122] Osteogenesis can be assessed in vitro by evaluating the cells' gene and protein expression profiles, differentiated morphology, calcium deposition, and level of activity of the Alkaline Phosphatase (ALP). ALP is one of the key substances that indicates whether or not the osteogenic precursors have entered the ECM deposition phase, a crucial factor that leads to the ultimate formation of new bone tissue. In fact, ECM proteins provide a substrate for bone cell adhesion and function, enabling the subsequent mineralization process. From a molecular point of view, many researches have focussed on analyzing the mitogen-activated protein kinase (MAPK) pathway, which is based on the interplay between a series of Ser/Thr kinases. These include extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), p38, and ERK5 families, all of which enable the functional regulation of specific transcription factors at the end of the phosphorylation cascade. This pathway is often involved in sensing the changes that are induced by magnetic forces in the microenvironments, cytoskeletons, cell membranes, matrices, and nucleoproteins.[123–125] Studies on animal models have focussed more on the stimulation of bone repair processes, such as tissue changes on a microstructure level, mineralization degree, acceleration of bone fracture healing, spinal fusion, and bone tissue ingrowth into ceramics. These phenomena have been predominantly assessed by testing the physical tissue properties (e.g., wound tensile strength, bone density).[126,127]

Meng et al. (2010) were inspired by the effects produced by MFs on bone wound healing; they proposed nanofibrous nonwoven films, which were manufactured by PVA electrospinning and contained γ-Fe_2O_3 NPs coated with meso-2, 3-dimercaptosuccinic acid and nHA.[128] The resulting construct was characterized by a connected porous network with average pore and fiber diameters in the range of 3–20 μm and 700–900 nm, respectively, and quasi-spherical MNPs with an average diameter of 14 nm. Besides promoting cell proliferation, the paramagnetic films induced a faster differentiation of a murine line that consisted of preosteoblast cells (MC3T3-E1) that had been cultured in an inductive culture medium for 21 days. The effect extent increased when an SMF (0.9–1.0 mT) was applied. When the control films were composed of only PLA and nHA, the cells maintained a fibroblast-like morphology. However, in the paramagnetic films, globular round cells integrated with the nanofibers were observed via Scanning Electron Microscopy (SEM); they were growing on the substrate and developing a thick substance (assumed to be the ECM) which slowly surrounded them. Some years later, these films were carefully folded, fixed to pellets, and then implanted in the lumbar transverse defect of rabbits, which were living in cages that had permanent magnets located on the opposite sides.[129] After 110 days, an in situ acceleration of bone tissue regeneration was demonstrated by computed tomography (CT) analysis and histo-pathological observations.

In 2014, another study reported that the magnetic energy inside MNP assemblies could enhance the differentiation of primary murine BM-MSCs in osteoblasts.[130] Here, topographical surfaces were fabricated by assembling bare γ-Fe_2O_3 NPs in stripe-like patterns on a glass surface in the presence of external MFs with field strengths ranging from 20 to 120 mT. This way, it was possible to regulate the cell behavior by means of the interface effect rather than the internalization effect. Even when ascorbic acid was present in the medium to initiate the process, the cell differentiation was affected by the magnetic coupling in the NP assemblies, positively correlating with the field strength of the external MF that was used in the assembly process. The authors proposed that the high gradient MF that arises from the remnant magnetic interaction inside the assemblies at the continuity break positions could be the cause of the observed phenomenon. Subsequently, a multitude of works showed that MNCs have the ability to prompt osteogenesis.[120,131–137] In 2016, Yun et al. discovered that PCL/MNPs composites and SMFs (15 mT) synergistically enhance the osteoblastic differentiation of primary mouse calvarium osteoblasts, as was proven by the increased expression of bone-associated genes (Runx2 and Osterix) and ALP activity.[120] The biomolecular analysis highlighted the up-regulation of bone morphogenetic protein-2 (BMP-2) and the phosphorylation of Smad1/5/8. The main mediators of the integrin signaling pathway were also activated, including: focal adhesion kinase (FAK), paxillin, RhoA, MAPK, and nuclear factor-kappa B (NFkB). Importantly, it was shown that this system could also indirectly affect other cell types by regulating the secretory function of the osteoblasts. The osteoblasts were, in fact, able to stimulate endothelial cells to express a vascular endothelial growth factor (VEGF) and angiogenin-1 genes, and to form capillary tubes. Wnt/β-catenin and BMP signaling pathways were also involved in the osteogenic differentiation of rat BM-MSCs that had been treated with magnetic graphene oxide (MGO, a novel combination of Fe_3O_4 and graphene oxide (GO)).[138] This material demonstrated a scavenging, ROS-regulating, and cytoprotective activity, as it decreased the oxidative stress occurring during the Fenton reaction, a process in which the ferrous iron from Fe_3O_4 reacts with hydrogen peroxide (H_2O_2) generating

hydroxide and hydroxyl radical. The coating rapidly eliminated a substantial amount of H_2O_2 and also reduced the Fenton reaction intermediate ·OH. By limiting the amount of cell damage caused by reactive oxygen species, the MSC activity improved. In a more recent study, MNCs that were based on oleic acid-modified IONPs and PLGA exposed to an SMF improved the cell attachment and the osteogenic differentiation of osteoblasts in a dose and time-dependent manner. This was proven by several biomolecular markers, including an increase in the ALP activity, mineralized nodule formation, and an upregulated expression of bone-associated genes (ALP, OCN, and BMP2).[139] Interestingly, a nano-deformation of the magnetic substrate developed under SMF, which was demonstrated via atomic force microscopy. This was claimed to be responsible for mediating the mechanical stimulation that triggered osteogenesis. The upregulation in the expression levels of a key receptor for sensing mechanical stimuli, known as piezo-type mechanosensitive ion channel component 1 (Piezo1), further corroborated this assumption. Recently, the time-dependent effects produced by MFs on the adhesion, proliferation, and differentiation of human MSCs were studied by Russo et al. in MNCs consisting of a PCL matrix and Fe_3O_4 NPs (80/20 w/w %).[136] In addition to observing an increase in the cell ALP activity, they also reported that applying the MF in a discontinuous manner strongly affected the phosphorylation of signal-regulated kinase (ERK)1/2. Some authors have also suggested that the hyperthermic effects of MNPs could be exploited for osteogenic regeneration.[131] A nanoheat stimulation method was proposed to exploit chitosan/polyethylene glycol (PEG) hydrogels, which incorporated uniformly dispersed Fe_3O_4 NPs. The high local temperature achieved by subjecting the gel to an AMF contributed to an increase in the osteogenic differentiation of the MSCs compared to the direct heat treatment applied under equal temperatures. Moreover, Tang et al. developed a method to magnetically and dynamically modulate the material surface properties in order to maximize the efficiency of certain cellular responses.[132] Their system consisted of an $CoFe_2O_4$/poly(vinylidene fluoride-trifluoroethylene) nanocomposite film, that displayed a surface potential variation ($\Delta V \approx 93$ mV) in response to the applied MF intensity (0–3000 Oe). It could operate in an in situ control of an integrin-adsorbed protein conformation, modulating the adhesion, proliferation, and differentiation of the cells via the FAK/ERK signaling pathway.

4.1.2. Tenogenic Differentiation

Magneto-mechanotransduction can also encourage tenogenic responses in SC cultures.[116,140,141] For example, tendon and ligament-mimetic scaffolds have been functionalized with MNPs to target cellular mechanosensor.[141,142] In one study, the magnetoactuation induced the anisotropic organization of the cytoskeleton in human ASCs (hASCs).[141] A mechanically reinforced and magnetically responsive substrate for cell growth was derived from a PCL matrix filled with rod-shaped cellulose nanocrystals decorated with IONPs. The diameter of the fibers (1.2–1.9 μm) fell within the range of the collagen fibers (1–20 μm), and their assembly resulted in continuous and aligned threads that matched the diameter of tendon fascicles (185 and 150–1000 μm, respectively). The stimulus conveyed by an SMF of 0.30 T steered the mechanosensitive signaling pathways, which were mediated by two transcriptional activators: YAP (Yes-associated protein); and TAZ (a transcriptional coactivator with a PDZ binding motif). In another work, hASCs labelled with MNPs were functionalized with anti-activin receptor type IIA (ActRIIA) before being combined with a fibrous aligned superparamagnetic scaffold. This scaffold was based on a biodegradable polymeric blend of starch and MNP-incorporated PCL.[142] Under AMF, the magnetic actuators synergistically triggered the ActRIIA, and a subsequent induction of the transforming growth factor-β (TGF-β) signaling pathway followed, through the Smad2/3 phosphorylation cascade. Consequently, the expression of tendon-related genes was augmented, along with the deposition, phosphorylation, and nuclear colocalization of the resulting proteins.

4.1.3. Neural Differentiation

Interesting investigations have started to focus on the preparation of magnetoelectric materials that can exploit the interaction between the magnetostrictive and piezoelectric properties of the NPs and polymers. Among polymers, polyvinylidene fluoride (PVDF) is one of the most promising in terms of its piezoelectric behavior. In one study, PVDF was electrospun together with nanostructures that were composed of $CoFe_2O_4$ nanoparticles (CFO) and GO sheets.[143] CFO was characterized by large magnetostrictive coefficients and high Curie temperatures[144] and served as a magnetostrictive phase, while GO prevented nanoparticle agglomeration. The resulting MNCs corresponded to an electroactive β-phase of PVDF, allowing the growth and differentiation of the MSCs to neural cells to be controlled noninvasively, without adding any differentiation factor, but only by applying extremely low frequency-electromagnetic fields (1 mT, 50 Hz) by means of a magnetic generator that had been placed inside the CO_2 incubator. The magnetoelectric nanofibers transduced the exerted MF of the bioreactor into a local electrical charge, which primarily enhanced the cell alignment. The analysis of neural markers, including Nestin (a cytoskeletal intermediate filament protein representing of self-renewal),[145] B-tub III and NSE (markers of an early and late commitment to the neural lineage, respectively),[146,147] and NGFR p75 (a transmembrane receptor of differentiated neural cells)[148] revealed that, in the presence of differentiation reagents (forskolin, retinoic acid and 3-isobutyl-1-methylxanthine), the rate of cell proliferation and differentiation decreased and increased respectively. However, applying a bioreactor for cell differentiation resulted in both cell proliferation and differentiation. In another study, Santhosh et al. incorporated MNP-decorated reduced GO in a collagen hydrogel.[118] By applying a low intensity external MF (≈50 mT) during gelation, the GO flakes aligned. Thanks to the excellent biocompatibility and electrical conductivity of the hydrogel, neuroblastoma cells (SH-SY5Y) displayed an enhanced differentiation and directionally oriented growth, with a propagation of calcium signal along the direction of orientation.

In 2019, Rotherham et al. demonstrated that Wnt signaling can be magnetically activated in the neuronal cell line SH-SY5Y, regulating the proliferation and differentiation of dopaminergic progenitors during neuronal development.[149] They exposed the cells to MNPs that had been functionalized with UM206 peptide, which binds to the Wnt receptor Frizzled. Under MF application (≥25 mT), the magnetic stimulation induced a β-catenin translocalization and activated a TCF/LEF responsive transcription element in the cells. This resulted in the expression of dopaminergic marker genes in the presence of the differentiation factors (retinoic acid and phorbol ester phorbol 12-myristate 13-acetate). The phenotypic response of cells was also maintained in in vivo models of the developing nigrostriatal pathway, demonstrating the translational value of such an approach.

4.2. Cell Proliferation

In addition to differentiation, other biological processes can be magnetically modulated. For example, cell proliferation is a rapid route for population expansion in unicellular organisms, and tissue growth in multicellular organisms. In this process, cells grow and divide to produce daughter cells.[150] While the proliferation of unicellular organisms is largely dependent on nutrient availability in the surrounding environment, cell proliferation in multicellular ones responds to gene regulation, which is partly controlled by signal transduction pathways that are elicited by growth factors during cell-cell communication.[151] During the first gap phase (G1) of the eukaryotic cell division cycle, cells decide whether to proliferate or remain quiescent. A number of signals (including growth factors, circulating hormones, developmental cues, and DNA damage) can trigger signaling pathways, starting the S phase, when DNA is replicated.[152] The G1-phase control is operated by a family of highly conserved proteins (i.e., Retino Blastoma proteins (pRB)), which negatively regulate the expression of the genes that encode the E2F transcription factors, which are required for entry into and progression through the S phase.[153,154]

In scaffolds that are constructed from MNCs, relevant effects are often observed on the growth rate of seeded cells.[110] In 2015, Daňková et al. produced nanofibrous scaffolds from a mixture of PCL and MNPs via needleless electrospinning, and then seeded porcine MSCs.[155] Augmented cell viability could be observed at one and three weeks after seeding. The authors claimed that, although the increase in the viability of cells cultured on an MNP-composite material had already been previously reported,[112,156] their work proved that such an effect can occur even in the absence of externally applied MF. By quantitatively monitoring the amount of DNA over time, they noticed that the cell proliferation rate increased as early as on day 1 of the experiment, then it gradually reached the maximal difference with respect to control groups on day 21. These results were in good accordance with other works in which accelerated cell proliferation occurred on nanofiber scaffolds made of a polymeric material coassembled with MNPs.[119,157,158] By acting as single magnetic domains on a nanoscale level, MNPs could affect ion channels and influence cellular processes. Moreover, they might exert mechanical stimuli directly by activating the mechanosensitive ion channels or deforming the cell membranes, subsequently initiating signaling pathways.[159] Importantly, it can be reasoned that the free iron released from the lysosomal degradation of IONPs could decrease intracellular H_2O_2 and change the expression of cell cycle regulators, causing cell cycle acceleration and promoting MSC proliferation.[160]

In 2011, Panseri et al. prepared a superparamagnetic bioactive phase by doping HA with Fe ions. Exposure to an SMF (320 mT) resulted in a significant increase in the cell proliferation throughout the experimental period, as well as more osteoblastic activity.[161] In constructs based on an Mg-HA-Collagen II matrix for bone regeneration, functionalization with maghemite NPs augmented the proliferation of hASCs by 70% compared to the control scaffolds, which were decorated with Au and Pd NPs,[162] whose use was previously associated with cytotoxic effects.[163–167] The increase in the proliferation was accompanied by an enhanced calcium deposition and an osteogenic differentiation of the cells. Given that these maghemite MNCs were studied in an absence of applied fields, the higher cell proliferation rate, as well as the superior osteoconductivity and osteoinductivity, was attributed to the high intrinsic MF of the superparamagnetic NPs. It has previously been demonstrated that γ-Fe_2O_3-based NCs have the ability to enhance the cell growth rate. For instance, in one study, 5% of γ-Fe_2O_3 NPs were mixed in a PVA matrix before being electrospun to form nanofibers.[168] Compared to neat PVA, the composite mats exhibited similar thermal properties and hydrophilicity, but also increased fiber diameter and surface roughness. As was assessed by an MTT assay, human skin fibroblasts (HSF1184 cell line) grew at an accelerated rate on the magnetic matrix (92% vs 80% onto the neat PVA). Wei et al. were also able to obtain magnetic biodegradable nanofibrous membranes, composed of chitosan and PVA and enriched with Fe_3O_4 NPs, via electrospinning.[157] The resulting matrix comprised a network of fibers with an average diameter ranging from 230 to 380 nm and a high porosity (84–85%). The MG63 human osteoblast-like cells cultured in this material displayed a high proliferation rate. The authors stated that the presence of MNPs generated several tiny MFs, which would subsequently locally exert osteoinductive effects. Assuming that each MNP acts as a single magnetic nanofield, it would develop a particular microenvironment in the pores or on the surface of the blend when it is incorporated into the matrix. The large number of magnetic microenvironments sensed by the cells increased the cell proliferation rate, while the nanophase augmented the cell area attachment thanks to its large surface area-to-volume ratio, enabling more cells to anchor. Other IONP-based MNCs have been prepared by using different matrix components, including other polymers and reinforcement nanoparticulates.[169] In order to continuously and steadily enhance the cellular activity, Bin et al. designed magnetic scaffolds composed of poly(L-lactide) (PLLA), which were prepared by selective laser sintering, and they were incorporated with 7% Fe_3O_4 SPIONs.[170] The constructs exhibited superparamagnetism and a maximum value of saturation magnetization of 6.1 emu g^{-1}. They promoted the attachment and diffusion of MG63 cells, favoured their proliferation, and prompted enzymatic activity typically occurring during osteogenic differentiation. According to the authors, each MNP in this system also constituted a single magnetic

domain, without a domain wall, becoming a micro-magnetic source that generated a tiny MF that was able to affect the cell behavior.

4.3. Cell Adhesion and Migration

Cell adhesion is the process by which cells physically interact with each other and with their substratum by establishing points of contact through specialized protein complexes.[171] While cell-to-cell adhesion is mediated by desmosomes, adherens or tight junctions, the cells interact with the ECM molecules through focal adhesions. Crucial effectors of cell adhesion are the transmembrane proteins that are located on the cell surface, and these are referred to as "cell-adhesion molecules" (CAMs).[172,173] In addition to signal transduction, which allows the cells to detect and respond to changes in their microenvironment, cell adhesion also regulates other processes, such as cell migration and tissue development. In particular, through its dynamic assembly and disassembly, the multi-protein complex that is formed in the focal adhesions (which links the filaments of the cell cytoskeleton to the ECM) enables the constitution of signaling complexes, driving cell growth and motility.[174]

By using long flexible PEG linkers, Kang et al. anchored SPIONs, functionalized with ligands, that contained the adhesive motifs Arg-Gly-Asp (RGD) to a glass substrate.[175] The SPIONs acted as adhesion nanoligands, performing an oscillatory motion that could be magnetically tuned by adjusting the frequency of an applied oscillating MF. A low oscillation frequency (0.1 Hz) allowed for integrin-ligand binding, thus promoting the formation and maturation of focal adhesions, while a higher oscillating frequency (2 Hz) inhibited the integrin ligation both in vitro and in vivo. A reversible regulation of SC adhesion was enabled by temporally switching the ligand oscillations between low- and high-frequency modes. Furthermore, the stimulation activated mechanosensing pathways and promoted differentiation in the same frequency-dependent manner. A few years later, Khatua et al. developed silica-coated SPIONs, conjugated to negatively charged RGD-containing ligands,[176] and distributed them onto a positively charged amino-functionalized substrate. The ligands are electrostatically coupled with the substrate surface. A reversible planar movement of the particles through the substrate was observed by applying an MF gradient. As a result, the nanosystems clustered in selected spatial areas. Since these NPs promoted human MSC adhesion through their externally exposed RGD elements, it was also possible to remotely and reversibly control the cell distribution both in vitro and in vivo by varying the NPs' macroscale density. This strategy allowed for the modulation of the integrin β1 ligation, focal adhesion number, cell adhesion, and spreading (assessed as adhered cell density and area, respectively). Notably, in magnetically stimulated zones, focal adhesions were characterized by the spread morphology, less aspect ratio, and pronounced vinculin expression. Moreover, such a ligand modulation system was able to promote mechanosensing-mediated differentiation toward osteogenesis. Importantly, the authors concluded the study by proving that cell adhesion could also be remotely regulated upon subcutaneous implantation in mice, with the effects lasting for 6 h. Since it is highly tissue-penetrative, the magnetic control is more translatable to in vivo applications than other biophysical triggers that have been thus far applied to the spatial modulation of cell adhesion, such as light or electrical fields. For instance, one group could spatio-temporally control the cell adhesion in vivo via UV light by using bio-adhesive peptides that could be activated upon removal of a protecting group via transdermal light exposure.[177] However, considering that UV light is massively absorbed by living tissue and can cause severe cytotoxicity, magnetic forces represent a safer alternative to control cells into living organisms.

Cellular adhesion is particularly critical in engineering vascular tissue grafts, especially with regard to a functional endothelialization of biomaterials. The endothelium represents an ideal biological component, and it has been identified as the only known completely nonthrombogenic material thus far.[178] Zhang et al. modified membranes made of bacterial cellulose (a natural biocompatible polymer with appropriate hydrophilicity for potential vascular use) with PEG-IONs, exposing the RGD motifs.[179] Enhanced adhesion and proliferation of murine endothelial cells (C166) on the substrate was observed. Furthermore, it was demonstrated that, by applying oscillating MFs with oscillation frequencies in a slow regime (0.1 Hz), a complete endothelialization of the graft could be reached within 4 days of culture, whereas higher frequencies (2 Hz) inhibited the cellular attachment. Finally, in addition to tuning cell adhesion, the MNCs were also proposed to regulate the cell migration, becoming a novel tool for magnetic guidance.[180] Magnetization (15 emu g^{-1} at 10 kOe) was applied to standard commercial scaffolds (composed of HA and collagen) by dip-coating them into aqueous ferrofluids that contained IONPs coated with various biopolymers. Through this procedure, the porosity and shape of the overall scaffold structure were preserved. Such magnetization was suitable to establish magnetic gradients, enabling the guidance of magnetized cells or materials (like growth factors) in the vicinity and inside the scaffolds. Thus, this strategy to guide cells and factors represents a possible solution for reloading the scaffolds with bio-agents after in vivo implantation.

4.4. Cell Phenotype Polarization

It was also proved that MNCs have the ability to fine tune the functional phenotype of specific cell types. For instance, nanofibrous superparamagnetic scaffolds and MFs were used to modulate the phenotypes of fibroblasts, promoting the emergence of a wound-healing profile in which the secretion of type I collagen (Col I), VEGF A, and TGF-β1significantly increased in a time-dependent manner.[181] The cells also released fewer pro-inflammatory cytokines, including interleukin-1β (IL-1 β) and monocyte chemoattractant protein-1 (MCP-1). Finally, additional changes in the secretory activity included an enhanced release of basic fibroblast growth factor (FGF), with an overall balanced production of the ECM components. The authors reported that the phenotypic polarization of fibroblasts was mediated by the activation of effectors in integrin, FAK, and ERK signaling pathways, as well as by the inhibition of the activation of Toll-like receptor-4 (TLR-4) and NFκB.

In 2017, Hao et al. provided the first evidence that it is possible to mechanostimulate the macrophage polarization toward an M2-like phenotype by inhibiting TLR2/4 activation while enhancing the VEGF Receptor 2 (VEGFR2) activation.[182] An increase in the production of osteoclast differentiation cytokines, such as the matrix metalloproteinase 9 (MMP-9) and the tartrate-resistant acid phosphatase (TRAP), was found in the cell secretome, along with an up-regulation of proinflammatory cytokines (IL-1β, TNF-α, and MCP-1), VEGF, and PDGF. In this study, the enhancement of osteogenesis and angiogenesis was attributed to the conditioned medium.

In another study, the expression of tendon-related markers in magnetically actuated hASCs was increased.[141] These cells also acquired a prohealing paracrine profile. Variations in their secretome included the upregulation of anti-inflammatory IL10 and IL4, and a lower expression of proinflammatory IL-6, as well as higher expression levels of MMPs and MMP tissue inhibitors (TIMPs). This indicated an enhanced remodeling response, a process that is known to be triggered by mechanical stimuli.[183]

4.5. Cell Death

Magnetic micromanipulation and stimulation on cellular structures can also induce cell death (**Figure 5**). One approach consists of mechanically stimulating membrane receptors in order to trigger the cell apoptosis signaling pathways.[99] Upon MF application, the death receptor 4 (DR4) could aggregate, then activate caspase-3, the effector caspase, and the death signal.[184] The mechanical sensitivity of other cell death-related receptors (such as FcεRI, PD-1, and PDL-1) has not yet been tested. Another route is the magnetic delivery of heat for hyperthermia, which can effectively raise the local tissue temperature, resulting in the eradication of target cancer cells, a decrease in tumor size and progression in vivo, and an enhancement of anti-tumor drug efficacy.[87,88] Finally, cell death can also occur as a consequence of the mechanical ablation of cellular structures via force or torque application.[185] For instance, rotating MFs can actuate the rotation of magnetic carbon nanotubes, rods, or particles in the cell, causing a mechanical disruption of its intracellular components.

4.6. Cell Metabolism

Mechanotransduction has a well-established role in controlling cell proliferation, differentiation and even death.[186] Since all these processes are energetically demanding, and they are dependent on the biosynthesis of various kinds of macromolecules, the mechanical forces and related signaling cascades also affect nutrient metabolism in cells. Indeed, a reciprocal crosstalk between cellular mechanics and metabolism exists, though the way in which they are connected is still poorly understood.[187] In magnetic actuation, metabolic alteration often occurs due to the changed cell activity.[188] It has been shown that the metabolic activity of aortic endothelial cells that have been seeded onto magnetite-impregnated alginate scaffolds becomes significantly elevated during the magnetic stimulation period.[189] However, their proliferation index was lower than that of the nonstimulated controls, which was proven by the expression of the proliferating cell nuclear antigen (PCNA). The authors suggested that the higher metabolic rate could correlate with cell migration and re-organization. However, it has to be reasoned that also the cell uptake, the intracellular trail, and the final fate of MNPs is strongly dependent on their specific formulation and anchorage to the scaffold matrix. Therefore, metabolic alteration could also be at least partially attributed to the interference of the actuators with the cell biochemistry. For instance, when the IONPs are internalized inside the cells, they can follow multiple endocytic pathways and enter different metabolic routes.[190] They can be preserved during cell life, then distributed to daughter cells during mitosis, or they can even be exocytosed out of the cells. However, they can also be degraded in the lysosomes so that the released free iron flows into the intracellular iron metabolic pool. Consequently, the cell iron metabolism can be affected, showing the upregulation of iron-related proteins (including the ferritin chains and ferroportin 1). As well as iron accumulation, the iron released from the MNPs can also potentially contribute to Fenton's reaction and produce reactive oxygen species that would damage macromolecules and organelles via oxidative stress.[191] Such deregulations could result in abnormal protein aggregation in the cells. Using MNPs that have been immobilized into polymeric scaffolds is a reasonable option to circumvent the potential toxicity risks and safely exert magnetic induction on cells in the proximity.

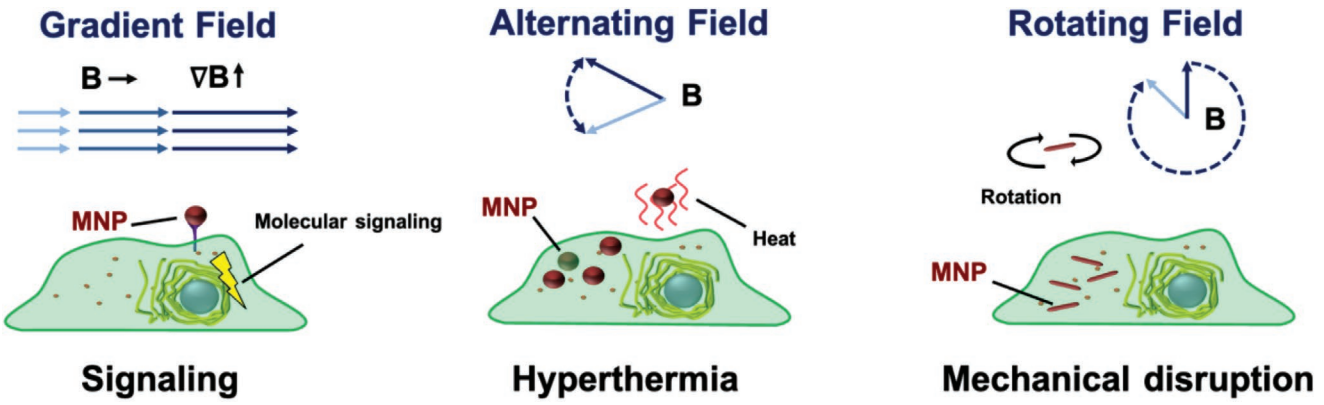

Figure 5. Magnetomechanical induction of cell death. Stimulation of mechanosensitive receptors on the cell surface for the activation of apoptosis signaling (left), hyperthermia-mediated heat generation (middle), and mechanical ablation using rotating magnetic tubes/rods (right).

5. MNCs for Tissue Engineering

MNCs are promising for applications in the engineering and repair of biological tissues. Implantable scaffolds for TE purposes based on MNCs have been presented.[12,119–121] In these scaffolds, MNPs can mediate topographical variations in the substrate or release bioactive agents that can affect cell growth. Furthermore, magnetic constructs can be used to magnetically apply a mechanical stimulus to the cells, promoting cellular differentiation in bone, cartilage, muscle and connective tissue.[192] Moreover, MNCs and MFs can be used to assemble multicellular constructs, orchestrate the spatial organization and guidance of cells, and prepare biomedical hybrid materials. Magnetic guidance can also be applied to navigate motile systems on a small scale (e.g., micro-robots) for tissue engineering and drug delivery applications.

5.1. Interactions of Magnetized Constructs with Cells

Implantable magnetic scaffolds can be used to replace damaged tissue since they can magnetically interact with seeded cells in a direct or indirect way (**Figure 6**). These magnetic scaffolds can be obtained by directly enriching the structure of a polymeric matrix with MNPs.

Direct cell stimulation mainly relies on the aforementioned mechanisms of magnetic actuation, which are operated by the magnetic forces and actuators.[12,193] While such magnetized constructs are involved in the engineering of neural,[194] cardiac,[195] and skeletal muscle tissue,[196] they have been exploited more often for bone repair.[11,12,119–121] This is due to the fact that magnetic actuation was shown to serve as an instructive mechanical cue for the activation of the mechanotransduction signaling pathways that result in osteogenic cell differentiation.[17] Furthermore, such MNCs have also been useful in the field of vascular engineering, in which the remote controllability of smooth muscle cells, endothelial cells, and fibroblasts is of utmost importance.[188,197–199] Endothelial cells, which had been loaded onto magnetized macroporous alginate scaffolds, were magnetically induced to form cellular vessel-like (loop) structures known as indicators of vasculogenesis and angiogenesis.[189,197] Remarkably, this effect was achieved by only subjecting the constructs to an AMF (40 Hz) for 7 days, without supplementing any growth or angiogenic factors. It was reported that, in the nonstimulated constructs, the cells only developed sheets or aggregates without any form of structured vascularization.[197]

Indirect stimulation occurs when magnetically responsive materials are used as scaffolds; the applied MFs induce physicochemical changes in the cells' microenvironments, which, in

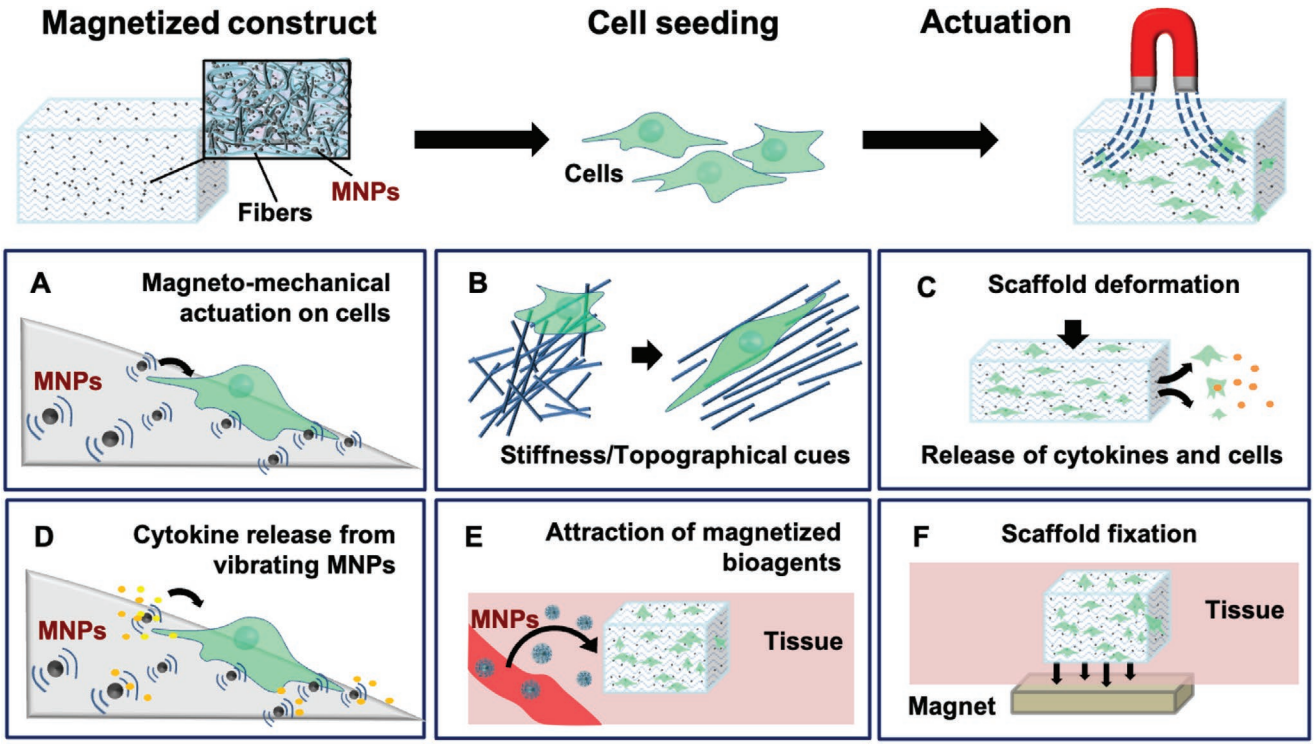

Figure 6. Implantable scaffolds based on MNC-magnetized matrices, and their use in TE. Under MF stimulation, each MNP in the scaffold acts like a single magnetic domain, causing micromotions at the cell-scaffold interface. A) This might affect the receptors and ion channels on the cell membrane, activating the mechanotransduction pathway. B) The incorporation of MNPs can increase the substrate stiffness or vary its topography, which, in turn, affects cell behavior. C) GFs and cells can be released following the magnetically induced scaffold deformation. D) Magnetically stimulated, cytokine-conjugated MNPs can locally release biological factors. E) Magnetized constructs can recruit magnetized bioagents (factors and cells) from the surrounding tissue and circulation. F) Magnetized scaffolds better integrate into the host tissue by means of magnetical fixation. Magnetic interactions between the scaffolds and cells can occur either in a A) direct or B–F) indirect way.

turn, affect the cell functions. One example is substrates whose magnetization results in modifying the scaffold's fine structure or topography, thus promoting cell adhesion and favouring supracellular organization into tissue-specific architectures. For instance, cell alignment occurs as a result of the magnetic orientation of the scaffold's constitutive fibers. Johansson et al. manufactured magnetic Ni-nanowires (200 nm in diameter and 40 μm in length) that adhered to glass cover slips and aligned when an external MF was applied (110–115 mT).[200] After a few days of culture on such a support, L929 fibroblasts or dissociated dorsal root ganglia neurons displayed contact guidance during growth, demonstrating that magnetically manipulating the substrates affects the spatial cell organization. In the hydrogels that were developed by Tognato et al.,[201] the IONPs could be aligned by low intensity MFs (20 mT) in a filamentous texture within a gelatin methacryloyl matrix. The cells that were seeded on top or embedded within the hydrogels oriented along the same axes of the NP filaments, and a differentiation of C2C12 skeletal myoblasts into myotubes occurred even in the absence of differentiation media.

Furthermore, magnetic scaffolds could also cause indirect stimulation by providing a controlled delivery of the growth factors that are necessary to stimulate cells over time and reach complete biological and histomorphological tissue maturation.[202] Even though MNCs (especially in the form of hydrogels) have been widely investigated as controllable drug delivery systems,[192,203–205] their use for tissue regeneration has thus far been limited. A recent example is the magnetically driven delivery system that was accomplished by Wang et al. In this system, drugs, proteins, and even cells could be extruded from the core part of the hollow fibers due to the deformation of 3D-printed alginate scaffolds under MF.[206] Furthermore, biofactor-conjugated MNPs, that vibrate when an MF is applied, can also release biochemicals on demand.[192] Alternatively, magnetized cells and biofactors can be recruited into the MNC scaffolds from the circulation or surrounding tissue, following local field gradients derived from the magnetization of the matrix.[180,192] Finally, the fixation of magnetic scaffolds in the defect site can also be enhanced with the help of an external MF. By doing this, macro and microscopic movements at the scaffold-host tissue interface could be avoided, resulting in a better integration of the constructs.[207]

Magnetized scaffolds can be actuated by applying external MFs of different types and characterized by diverse parameters (such as frequencies, amplitudes, and duration). SMFs and PEMFs are used most widely in magnetic actuation. In general, the MFs can be provided as either gradient or uniform fields, and have frequencies below 1 MHz, as low frequency MFs penetrate living tissues without limitations. Nevertheless, the selection of an actuation strategy heavily depends on the specific application and the target tissue. In addition, the nature of the magnetic component, as well as the scaffold's composition and design contribute to differently condition the biological effects observed.[208]

The field gradient imposes volumetric forces on the actuatable material, that could be an MNP attached to the cell membrane or a magnetized scaffold. Time-varying gradient MFs allow the scientists to manipulate the MNPs attached to the cell membrane, activating the receptor-mediated signal transduction and mechanically conditioning the cells for regenerative application.[99] The magnetic forces involved fall in the piconewton range.[209] Gradient MFs are also useful for drug release from magnetic substrates, or stimulation of the polymeric MNCs to modify the scaffold architecture and influence tissue regeneration.[210,211] Even though gradient fields are commonly employed, their in vivo implementation is technically challenging as the gradient field magnitude depends on the size of the field-creating device and the distance to it.[212]

Time-varying uniform MFs generate transient physical forces into MNP-embedding 3D scaffolds. These forces can arise from reversible scaffold shape deformation due to alignment of scaffold's walls to the applied field, and are transferable to cells located in close proximity to the MNPs. In particular, alternating cycles of alignment and relaxation in the scaffold structure cause bending/stretching forces that exert a mechanical action on the cells. Interestingly, the estimated mechanical force that can be imparted on cells (in the order of 1 pN) correlates well with the threshold value to induce mechanotransduction effects on cellular level (0.2 pN).[212,213] AMFs coupled to anisotropic magnetic scaffolds can also induce mechanical vibration of small magnitude that can affect the cells' structure and processes, like the viability, proliferation, adhesion, and differentiation.[214–216] AMF-induced oscillation of MNPs can also mechanically damage the cells and be used for cancer treatment.[217,218]

Another relevant factor to take into account when selecting the actuation approach is the target tissue. Magnetic actuation for bone tissue regeneration is often performed in the presence of MFs with moderate intensities varying in the range 1 mT–1 T, as these fields have demonstrated to promote osteogenesis by enhancing preosteoblasts proliferation, ECM production, and mineral template deposition.[219] Low-frequency MFs (20 mT, 1 Hz) were found to promote neuronal differentiation and modulate synaptic functions,[220] and homogeneous sinusoidal extremely low-frequency MFs (5 mT) improves the growth factor-induced chondrogenic differentiation of hMSCs.[221]

5.2. Spatial Guidance of Magnetized Cells

When the cells are labelled with a core–shell type MNC (such as a polymer-coated MNP), they can also respond to external magnetic guidance, which causes their physical displacement or orients their natural motility.[222] The magnetic spatial control of cells can offer several advantages in tissue regeneration (**Figure 7**). One possibility is to use MFs to enhance the retention of magnetized cells in target destinations in the body after they have been systemically administered.[97,223–227] Such an approach also succeeds in enhancing and directing the movements of cells in intratissue transplantation; therefore, it is useful in terms of increasing the migration efficiency of transplanted magnetized SCs toward injury sites.[222] In general, magnetized cells injected within a body can be guided by SFMs applied as gradient fields.[228,229] The amplitude of the applied MFs depends on the operation location, as well as the type of magnetic materials, their morphologies and sizes.

Alternatively, in a method referred to as "magnetic 3D bioprinting," the cells are incubated with a biocompatible NP

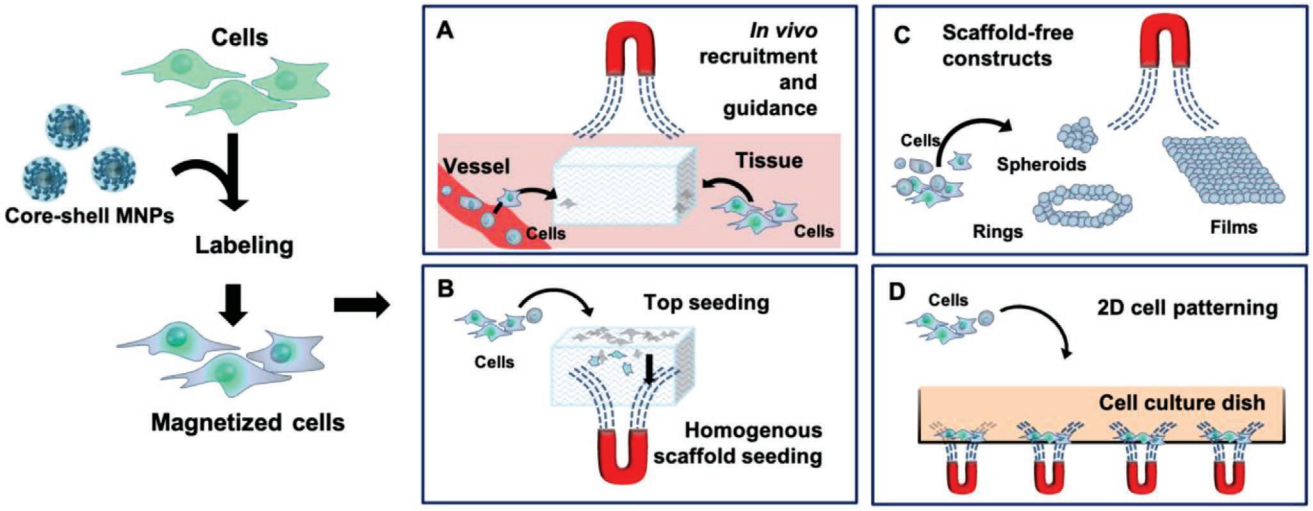

Figure 7. Constructs based on magnetized cells, and their use in TE. The cells become magnetized when they are labelled with MNPs. Magnetized cells can be: A) recruited in vivo at a specific target site (scaffold, wound, injured tissue) following systemic injection or intratissue transplantation; B) guided through 3D scaffolds, leading to homogeneous cell distribution; or C) assembled into scaffold-free 3D structures via magnetic levitation or 3D bioprinting. D) Cell patterning can be achieved by differential spatial distribution of the MF strength in substrates for cell growth.

assembly (consisting of gold, iron oxide, and poly-L-lysine), and they become magnetized.[230–236] After, mild magnetic forces (i.e., magnetic field strengths below 0.5 T) levitate the cells in the culture media, which means they can be rapidly printed with a high reproducibility in specific 3D configurations. Constructs with complex shapes can be generated through magnetic force-based tissue engineering (Mag-TE),[199] a method that has been exploited to engineer not only 3D scaffolds[237] and cell sheets,[121,238] but also tubular structures, which are of particular interest for engineering vascular tissue.[199,239,240]

Upon labelling with MNPs, cells that have been cultured on ultra-low attachment plates can be exposed to SMFs and assembled into cell sheets.[199] Subsequently, the sheets can be rolled onto a cylindrical magnet, forming a tube around it. This approach has been applied to heterotypic layers of endothelial cells, smooth muscle cells, and fibroblasts to form vascular tissue. Furthermore, it has been demonstrated that MNP-labelled cells can biophysically interact with flexible magnetic sheets, remaining stably attached at the surface while undergoing simulated blood flow rates of up to 300 mL min^{-1}, with cell loss commencing at 400 mL min^{-1}. Potential applications of this technique in a rapid endothelialization of synthetic vascular grafts and dialysis fistulas have been hypothesized.[198]

Finally, magnetically actuatable scaffolds in a tube-like shape have been obtained by winding electrospun sheets of a biodegradable polymer modified with Fe_2O_3 NPs. The tubular scaffolds were seeded with smooth muscle cells, and actuated by an MF. This caused a cyclic crimping deformation, which induced a strain stimulus in the cells. A nutrient fluid was pumped through the porous tube walls, ultimately increasing the cell proliferation.

5.3. Magnetic Biomedical Microrobots

By expanding the definition of polymeric MNCs to include the combination of MNPs with complex systems, magnetically controlled microrobots or nanorobots can also be considered a pertinent multimaterial[241] with substantial applicative potential in tissue regeneration. In fact, MNPs and other magnetic materials can be used to navigate synthetic or natural micropropellers or nanopropellers, while MFs can provide both a driving force for propulsion and a mechanism for steering (**Figure 8**).[242–244] Such robots are able to swim through three different configurations: MNPs can be driven by MF gradients; magnetic rod structures can beat in a transverse oscillatory movement; and rigid particles can be driven by rotating MFs.[245] The various magnetic actuation designs of microrobots have already been reviewed by Yu et al.[246]

In the field of biomedicine, most conventional applications of micro/nanorobots focus on a targeted delivery of chemicals and cells,[247] and the assistance of intercellular dynamical processes such as fertilization.[248] Porous matrices that enable a targeted drug delivery from microrobots have been either artificially manufactured,[249] or derived from natural sources (bacteria, fungi, pollen).[250–252] Recent works have focused on the synthesis of biocompatible and biodegradable polymers for micro/nanorobots dedicated to in vivo use.[253] For example, Park et al. presented a porous degradable microrobot, which was magnetically actuated by rotating MFs, that consisted of a helical soft polymeric (PEG diacrylate and pentaerythritol triacrylate) chassis that contained both magnetite NPs and an antitumor drug.[252] Various microrobots have been synthetized and magnetized in order to move inside animal bodies, localize at a target site, and deliver regenerative cells to the injured tissue, all under magnetic control.[12,247,254–256] Their real-time localization can be achieved via MRI due to their intrinsic magnetic contrast ability.[250,252]

In the near future, applications in TE are expected to become a vigorous research area. Magnetically actuated robots have the potential not only to become bio-scaffolds that can support tissue regeneration,[255–257] but also to orchestrate the assembly of microscaled tissue building blocks and to construct living

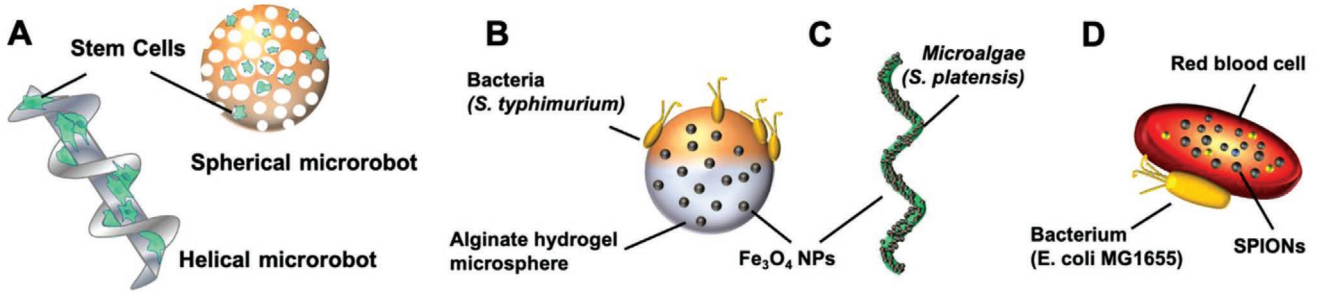

Figure 8. Magnetic microrobots for biomedical applications and TE. The layout of helical and spherical scaffold-type synthetic microrobots for delivery of SCs in vivo. A) The structures are coated with nickel and titanium layers for magnetization and biocompatibility, respectively. The design of magnetic biohybrid robots: flagellated bacteria, with attenuated toxicity and genetically induced fluorescence expression selectively, are attached to one side of chitosan-coated alginate microspheres. B) One microparticle side was treated with O_2 plasma, making it hydrophilic, thus preventing bacterial attachment. C) A microrobot, based on a helical microalgae, following dip-coating in a suspension of Fe_3O_4 NPs. D) A microswimmer, composed of a red blood cell, loaded with drug molecules and SPIONs, bound to a motile bacterium via a biotin-avidin-biotin binding complex.

tissues in vitro.[258] For instance, magnetic micro crawlers and microgrippers could be used to assemble microsized hydrogels (microgels) laden with different cells.[259–261]

Nevertheless, to realize the full potential of miniaturized robots for medical applications, strategies are needed to ensure a safe interaction with the human body. In this scenario, a subcategory of robots, termed "biohybrids," has emerged due to the remarkable biocompatibility and degradability of their constituent materials. Magnetic biohybrid microrobots are realized by associating magnetic particles (often MNPs) to structured, functional, and even actuated cellular materials, including entire eukaryotic or bacterial cells.[250,262–265] For instance, biological propulsion in spermatozoids, bacteria, protozoa, and microalgae, as well as the contractility of skeletal muscle and cardiac cells, is orchestrated by macromolecular machines that can, in principle, be adapted through the use of magnetic materials to respond to steering control from externally applied MFs.[265–267] Biomimetic and non-biomimetic microrobots can be magnetically guided via various methods employing rotating fields, oscillating fields, or field gradients, that can be classified into force-driven or torque-driven actuation approaches.[268] Despite the great potential of magnetic biohybrids, their use entails some concerns with regard to the pathogenicity, the production of hazardous by-products, immunogenicity during use, and the risk of microbial contamination during fabrication.[269] These issues could possibly be addressed in the future by applying sterilization strategies and genetical engineering to eliminate pathogenicity and control the cellular processes,[270] and by employing patient-derived induced pluripotent stem cells (iPSC) to evade a negative immunological response.[271]

5.4. Preclinical Test of MNCs

As shown in the previous sections, MNCs and MFs have demonstrated an evident ability to modulate the cell behavior in controlled experimental conditions in vitro. The conclusions from cell experiments were also often corroborated by proof-of-concept studies carried out in preclinical models. Indeed, several researches on the MNCs focused the conceptual validation of the tissue repair in animal models. To confirm in vitro data, in vivo imaging techniques are adopted to monitor the tissue evolution over time, whereas ex vivo histological analysis provides additional information with higher spatial and molecular resolution. Thus far, MNCs have been predominantly implanted in small mammals, namely mice, rats, and rabbits.[120,133,139,272–274]

In bone regeneration, the use of magnetized hydrogels or scaffolds in both orthotopic and ectopic models had repercussions that were visible at the tissue level as variations in collagen deposition, matrix mineralization and remodelling, host cell infiltration, and frank bone tissue formation.[117] For instance, when PEG-hydrogels enriched with MNPs and seeded with stromal vascular fraction cells were magnetically preconditioned in vitro and then implanted subcutaneously into nude mice, a highly mineralized and densely vascularized tissue formed along 8 weeks of in vivo ectopic development, as shown in **Figure 9**A.[275] MNCs have also been implanted into orthotopic defects models, as these implantations provide a physiologically relevant environment for testing the performance of regenerative therapies. Nanofibrous magnetized scaffolds were inserted in a rabbit model of lumbar transverse defects and kept for about three months.[128] In this time frame, μCT imaging revealed that the magnetic biomaterial and the external SMF acted synergistically to enhance the local bone reconstitution (Figure 9B). In fact, it was possible to visually determine that newly formed tissue had a homogeneous morphology closely resembling the one of the natural bone. Such an observation was confirmed by ex vivo μCT imaging and histological data gathered at a later time point (110 days), which suggested that, as compared to unactuated controls, the magnetically actuated scaffolds underwent a faster bone tissue remodeling through the complete adsorption of the scaffolding material and the formation of new bone tissue.[128] Another group of scientists used MRI to monitor gelatin sponges loaded with superparamagnetic NPs after implanting them in the incisor sockets of the Sprague–Dawley rats (Figure 9C). After four weeks, they noticed more newly formed bone and preserved alveolar ridge than in the blank controls.[276]

Magnetic guidance of nonself propelled cells upon labeling with MNPs has been mainly used to localize cells to specific target sites following systemic injection. In such a way, the cells

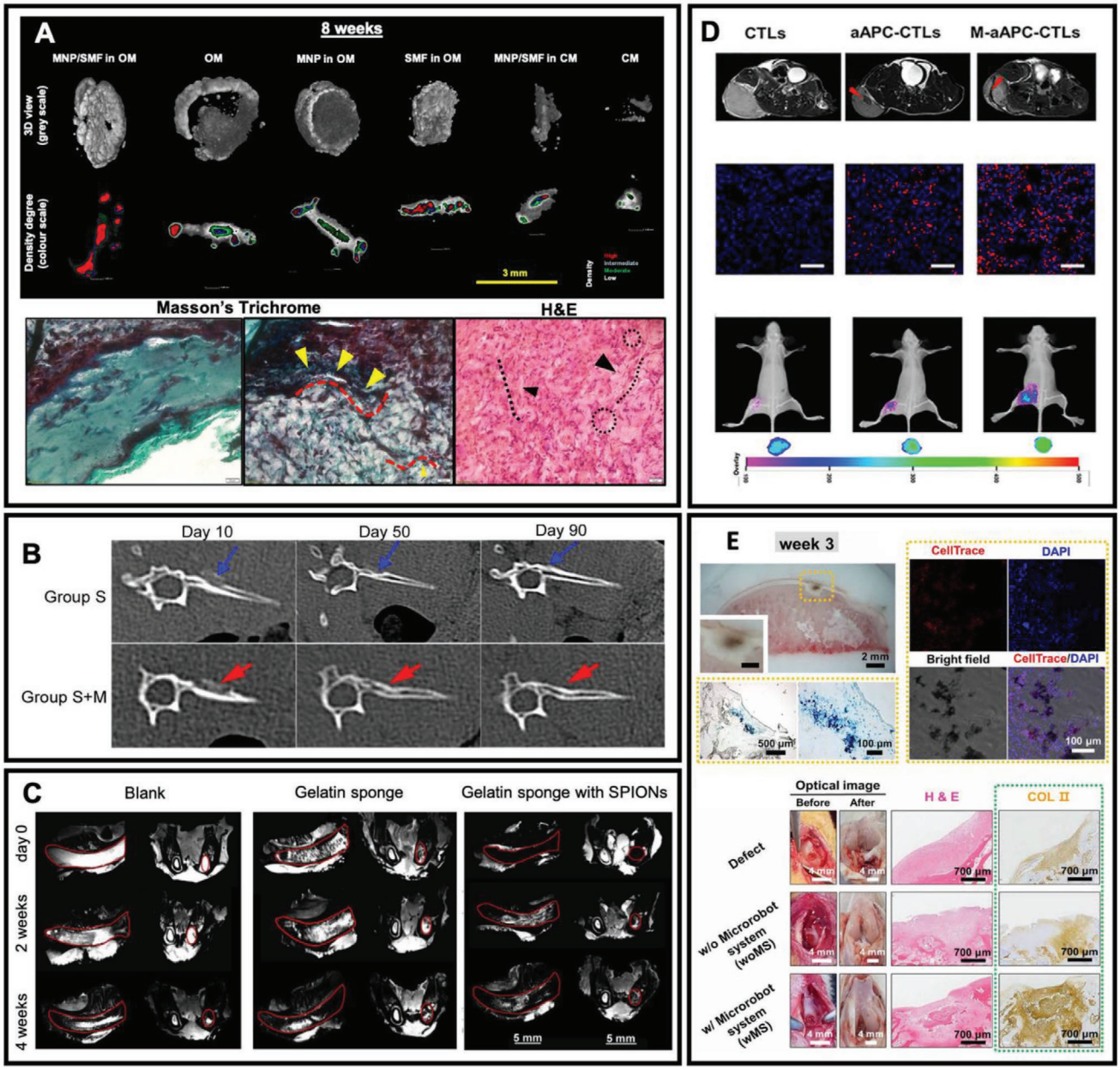

Figure 9. A) Preclinical applications of MNCs. MNCs generated from MNPs-incorporating hydrogels were cultured in culture or osteogenic medium (CM and OM, respectively), and exposed to SMF in vitro. MNCs were then subcutaneously implanted into nude mice and extracted after 8 weeks. Representative μCT imaging revealed dense tissue formation (top), whereas the histological analysis (Masson's Trichrome and H&E stainings) showed collagen deposition, cortical bone tissue-like formation, and numerous functional vessels (bottom). Adapted with permission.[275] Copyright 2019, Elsevier. B) CT imaging of rabbit lumbar transverse bone defects implanted with a nanofibrous composite scaffold in the absence (group S) or presence (group S + M) of an external SMF. Adapted with permission.[128] Copyright 2013, Springer Nature. T_2-weighted MRI of rat incisor sockets after the implantation of a scaffold composed of SPIONs-loaded gelatin sponge. C) After 4 weeks, more newly formed bone and a better preserved alveolar ridge were evident in the implantation site as compared to that of the blank and sole gelatine controls. Adapted with permission.[276] Copyright 2018, John Wiley and Sons. D) Magnetic guidance of cytotoxic T-cells (CTLs) incubated with biomimetic magnetosomes (aAPC) in a murine lymphoma model. MRI (top) showed higher tumor infiltration by aAPC-CTLs (indicated by the darker area, red arrows) than CTLs alone. Once a MF was applied (M-aAPC-CTLs), more aAPC-CTLs localized to the tumor. MRI data were confirmed by confocal microscopy (middle) and Near InfraRed imaging (bottom). Adapted with permission.[277] Copyright 2017, American Chemical Society. E) Microrobots loaded with human ASCs were injected and magnetically guided in the knee cartilage tissue defect in a rabbit model. Histology with Prussian Blue staining and fluorescence imaging of engrafted cells (marked in CellTrace, red) (top) and H&E and COLII staining of the cartilage tissue in defect group and microrobot system groups (bottom) at 3 weeks postinjection. Adapted with permission.[255] Copyright 2020, The American Association for the Advancement of Science.

conveyed by circulatory flows can be magnetically retained at the site of interest by allocating permanent magnets for cell attraction in predefined positions. Cytotoxic T-cells incubated with biomimetic magnetosomes were magnetically guided in a murine lymphoma model, while being monitored by fluorescence imaging and MRI (Figure 9D). As compared to

nonmagnetized controls, under MF application, the magnetized T-cells were capable of higher tumor infiltration. The strong cell localization to the tumor site was congruent to the observation that such a treatment consistently reduced the tumor growth.[277] Finally, magnetically responsive scaffolds loaded with stem cells can serve as microrobots that, upon in vivo injection, can be guided to a specific target site by locally applying MF gradients. Go et al. prepared microrobots designed as hollow spherical cages to transport ASCs and injected them in a rabbit model of knee cartilage defect.[255] The microrobots were manufactured through a sequential process in which magnetic microclusters were adsorbed on PLGA microscaffolds before the cell loading. The microrobots were guided in vivo to the target site through an electromagnetic actuation system consisting of multiple electromagnetic coils, and then immobilized to the damaged cartilage using a permanent magnet. The in vivo study was carried out in rabbits in order to obtain clinical trial approval, therefore all the employed technical tools were designed by taking into consideration the accessibility of the patient and medical staff, as well as clinical safety. The engrafted microrobots degraded in 3 weeks, as shown by histological gross observation and by the Prussian Blue staining which marks the iron deposits (Figure 9E, top left). However, the loaded cells did not disappear with the degradation of microrobots but migrated to the cartilage lesion site and engrafted in the tissue (Figure 9E, top right). To assess the effects of the microrobot system on the cartilage regeneration, the histological analysis was performed at the same time point, revealing a strong Collagen Type II expression (Figure 9E, bottom).

Here, we have provided some examples of in vivo tests performed in different animals and through different transplantation approaches (ectopic vs orthotopic), and assessed by diverse imaging techniques. Establishing magnetic actuation in vivo poses many technical challenges as it requires one to adapt the stimulation setting to a complex living environment. Nevertheless, in order to define the potential medical utility, MNCs have to be tested within bodies with an active physiology, especially if they are dedicated to tissue regeneration and in vivo imaging.

6. Discussion and Future Perspectives

The exceptional properties of MNPs renders them promising candidates for use in many domains, such as biosciences and electronics. In particular, over the last three decades, they have proved to be formidable in the field of nanotechnology with regard to interacting with living systems. Furthermore, since their intrinsic properties are often combined with a low immunogenicity and high biocompatibility, they have the potential to address current hurdles in biomedicine by overcoming the technical limitations that are found in material engineering. Combining them with polymers resulted in MNCs that could serve as TE scaffolds, as well as coatings, biosensors and pharmaceutical carriers.[53,278,279] Magnetic traction forces can induce the spatial displacement of physical entities over a wide scale range (from the nano to centimeter); therefore, they can be employed for cell, organelle, or macromolecule guidance. Indeed, applications have already explored various fields, such as genetic engineering, signaling modulation, cell seeding and patterning, and in vivo cell targeted delivery.[23] Notably, having observed that MNPs and MFs can modulate cell functionality to some extent,[92–95] magnetism is now expected to aid the development of remote control SC techniques. Nevertheless, even if several reports indicate that IONPs have limited adverse effects on cell behavior,[91] their exact impact on SC functions has still to be clearly elucidated. In recent years, increasing evidence has proved that magnetic actuation technologies are effective at harnessing MSCs and other progenitor cells toward bone, cardiac and vascular destiny.[149,189,195] In particular, a multitude of MNCs for bone TE have been designed as films, scaffolds, and implants to meet different needs.[280] The value of these approaches lies in the ability to control the SC differentiation process by acting on specific molecules. Such technical precision allows the cell functions to be finely tuned. Nevertheless, even if magnetic actuation can stimulate the cell mechanosensing system and promote SC differentiation, magnetic actuation has often been applied in combination with other differentiation-inducing techniques (e.g., biochemical factors),[128–30,182,280] and there are very few reports of cell functional control under pure magnetic stimulation.[143,197,201]

Since the spark of regenerative medicine fuelled the interest in controlling the repair process, the predominant approaches to achieve the phenotypic modulation of SCs have been: small molecules that target the intracellular pathways; genetically manipulating the cells; and engineering the bio-physical properties of the matrices.[281–287] In parallel, several types of smart dynamic biomaterials have also been developed to stimulate regenerative cells:[288,289] surfaces endowed with stimuli-reacting properties (such as photo-actionability, electro or thermo-responsiveness), and enzymatic sensitivity can both support cell self-renewal and differentiation with spatiotemporal control.[79,155,160,290,291] The possibility to regulate the tissue healing process at a clinical level by simply applying external MFs is an extremely appealing objective, and the body of literature concerning the role of magnetism in affecting cell behavior is continuously expanding. However, the collected results strictly depend on the experimental conditions and cell types used, and the principles of cell stimulation within magnetically responsive matrices still need to be precisely elucidated at a cellular and molecular level.[73,124,292] Moreover, a rigorous predictive approach for MNC classification according to the distinctive resulting magnetism is still missing, and a general underlying theory can hardly be formulated, since the MNC magnetism is critically dependent on the type of NPs, host matrices, and manufacturing techniques that are used.[64,65] Therefore, even though remotely harnessing the SC function through magnetism is tremendously fascinating,[293–296] there is still a lot of work to be done before these concepts can be validated with human cells from various sources. Ideally, magnetic actuation should demonstrate capability to form tissues that can ultimately achieve successful integration into patients' bodies. Encouragingly, the first studies for developing technologies of clinical relevance have started,[293] even if the postimplantation magnetic stimulation of exogenous SC populations poses a notable translational hurdle. The urgency to gain a better understanding of the underlying biological mechanisms

is motivated by the increasing amount of newly introduced paradigms and models for magnetic actuation.[297] Nevertheless, the benefit of MNCs in SC modulation opens a gateway to new research into sophisticated and innovative approaches to overcome the current limitations of engineered tissues and strategies for their repair (such as 3D cell seeding and patterning, the microenvironment control, and the functional modulation of cell behavior).

Several biomedical MNCs have been developed in the form of anisotropic magnetically responsive hydrogels that demonstrated the ability to engineer different tissues of the human body.[298] As biomaterials, the MNCs show tunable response to the MF exposure, which depends on the properties of the magnetic and the polymer phases, but also on the parameters of the applied MFs. Here, we have shown that the combination of MNCs with living systems can have repercussions on various cell activities. In the future, materials with higher magnetic responsiveness could be used to decrease the amplitude of the MFs required for MNC manipulation and improve the controllability of engineered tissues, but also to reduce the amount of incorporated MNPs and minimizing the risks of potential MNP-related cytotoxicity.

7. Conclusions

In conclusion, MNCs have been consistently applied in a controlled release of therapeutics to treat various diseases,[192,204,205] but more research is still needed to properly optimize such systems for use in tissue regeneration. Strategies to deliver growth factors and cells, which can biologically and chemically affect tissue development, will play a crucial role in upcoming fundamental and applied research. Moreover, advancing magnetic responsive materials that could directly control cellular behavior will drive the exploration of remote control of tissue development. In the near future, MNCs are expected to have impactful implications in the methods for cell culture, the construction of complex cellular assemblies, and the actuation of biohybrid materials. As discussed in this review, the MNCs are highly biocompatible and can positively affect tissue maturation in vivo, which renders them very promising for tissue repair. Finally, we have shown that the intersection between biological and mechanical engineering disciplines has generated robotic agents whose propulsion can be controlled and directed by magnetism. Indeed, in the last decade, it has become clear that, besides serving for the construction of guidable passive delivery systems, MNCs are also excellent materials to create microscaled robots endowed with autonomous motion ability, that can be remotely oriented by magnetic forces.

All of these implementations suggest that the potential of the MNCs in biomedicine is wide-ranging, although many aspects of their interaction with living systems are still quite unexplored. The authors expect to witness the surge of exciting achievements in this field in the near future.

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Conflict of Interest

The authors declare no conflict of interest.

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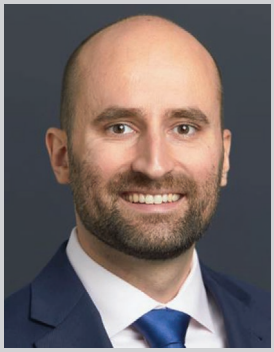

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