Biomedical Materials

REVIEW

Magnetic responsive cell-based strategies for diagnostics and therapeutics

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Abstract

The potential of magnetically assisted strategies within the remit of cell-based therapies is increasing, creating new opportunities for biomedical platforms and in the field of tissue engineering and regenerative medicine. Among the magnetic elements approached for building magnetically responsive strategies, superparamagnetic iron oxide nanoparticles (SPIONs) represent tunable and precise tools whose properties can be modelled for detection, diagnosis, targeting and therapy purposes. The most investigated clinical role of SPIONs is as contrast imaging agents for tracking and monitoring cells and tissues. Nevertheless, magnetic detection also includes biomarker mapping, cell labelling and cell/drug targeting to monitor cell events and anticipate the disruption of homeostatic conditions and the progression of disease. Additionally, the isolation and screening techniques of cell subsets in heterogeneous populations or of proteins of interest have been explored in a magnetic sorting context. More recently, SPION-based technologies have been applied to stimulate cell differentiation and mechanotransduction processes and to transport genetic or drug cargo to study biological mechanisms and contribute to improved therapies. Magnetically based strategies significantly contribute to magnetic tissue engineering (magTE), in which magnetically responsive actuators built from magnetic labelled cells or magnetic functionalized systems can be remotely controlled and spatially manipulated upon the actuation of an external magnetic field for the delivery or target of TE solutions. SPION functionalities combined with magnetic responsiveness in multifactorial magnetically assisted platforms can revolutionize diagnosis and therapeutics, providing new diagnosis and theranostic tools, encouraging regenerative medicine approaches and having potential for more effective therapies. This review will address the contribution of SPION-based technologies as multifunctional tools in boosting magnetically assisted cell-based strategies to explore diagnostics and tracking solutions for the detection and analysis of pathologies, and to generate improved treatments and therapies, envisioning precise and customized answers for the management of numerous diseases.

1. Introduction

Advanced cell-based strategies will benefit from emerging knowledge on the magnetic responsiveness and superparamagnetic iron oxide nanoparticle (SPION) functionalities applied to clinics, boosting the development of magnetically actuated systems to meet improved solutions for biomedicine and regenerative medicine demands.

SPIONs have been explored for a wide range of biomedical applications, including magnetic resonance imaging (MRI), hyperthermia and targeted drug delivery [1–4]. Under the controlled and precise actuation of a magnetic field (MF), SPIONs (and

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consequently, SPION incorporating systems) are guided and concentrated at the desired site. The principle behind the unique features of SPIONs relies on their superparamagnetic behaviour, whose magnetic responsiveness only persists as long as the MF is being applied, allowing a real-time non-invasive follow-up treatment. Moreover, magnetism-based approaches allow the remote tracking, control, monitoring and/or stimulation of SPIONs and/or SPION-incorporating systems, offering new perspectives and solutions either in diagnostics or therapeutics.

In cell-based strategies, SPIONs with a magnetic core of magnetite (Fe_3O_4) or maghemite (Fe_2O_3) are often polymer coated to improve their biocompatibility and their structural and colloidal stability, while providing functional groups for bioactive molecule conjugation [5] and/or ligands for targeting cells or tissues.

SPIONs are considered to be non-cytotoxic under 100 μg ml^{-1}, easily cleared via endogenous metabolic iron pathways and metabolized into its elements [6] and/or eliminated by kidney excretion [7]. Many studies have investigated SPION uptake by different cell types without evidence of a pejorative cellular response [8, 9], suggesting the applicability of these particles for bioengineering and regenerative medicine approaches.

Smart systems integrating SPIONs anticipate the development of detection platforms able to image, guide and activate therapeutic cells combining diagnostics and therapeutics. Moreover, magnetically assisted systems can also be applied in pharmacotherapy as cargo vehicles of bioagents or drugs, assisting the development of predictive *in vitro* disease models to be further used as screening platforms of candidate treatments toward patient customization therapies.

Magnetic actuators also enable cell-based therapies to be guided by finely tuning the cell phenotype and behaviour influencing proliferation, differentiation and selective protein expression with magnetic actuation [10–12], or via the magnetic-enhanced transfection of genes of interest [13]. In this sense, SPION technologies hold potential in the design of biomolecular solutions with a direct role in biological processes, influencing or correcting the biological mechanisms involved in abnormal or undesired responses that lead to disease or to failure in the regeneration process, thus envisioning a new window of opportunity for customized treatments and improved healthcare.

In summary, SPION-based technologies constitute a holistic, integrated and versatile platform with potential diagnostic and therapeutic actions to manage numerous diseases and revolutionize regenerative medicine, anticipating personalized medicine solutions (figure 1). This review will address the different directions of SPION-based technologies as multifunctional tools in magnetically assisted diagnosis and therapeutic strategies, focusing on tissue engineering and regenerative medicine applications.

2. SPIONs as diagnostic tools for cell-based therapies

The development of effective treatment for injury or disease could benefit from combinations of suitable imaging and detection methods for differential diagnosis. Diagnosis is often challenging and demands complementary and accurate information to allow personalized medical decisions about treatment and prognosis to be made.

SPIONs have been explored as MRI contrast agents and as biosensors for the identification of cells, tissues and biomarkers associated with both healthy and diseased conditions (figure 2).

2.1. Imaging

Medical imaging techniques such as MRI, x-ray-based computed tomography (x-ray CT), positron emission tomography (PET), single photon emission computed tomography (SPECT), fluorescence imaging and ultrasound have become available to image the anatomy and physiological processes of the body, both healthy and diseased. Among them, MRI is a powerful medical diagnostic tool that lacks ionizing radiation and allows one to non-invasively image cells and tissues with excellent resolution and excellent tissue penetration depth [14]. To increase the sensitivity of MRI images, contrast enhancing agents are commonly used. These can be divided into T1 and T2 contrast agents. The first shorten the longitudinal relaxation times resulting in the brightening (positive contrast) of surrounding tissues, whereas the latter shorten the transverse relaxation times resulting in darkened (negative contrast) areas.

Gadolinium (Gd)-based T1 contrast agents were the first generation of contrast agents used to brighten tissues in MRI images. However, emerging research suggests that these agents may accumulate in the brain and have been reported to induce nephrotoxicity [15, 16]. SPIONs, primarily used as negative (T2) contrast agents, have shown the potential to replace Gd-based contrast agents due to their lower toxicity and improved biocompatibility and became the second generation of MRI contrast agents [17]. Several SPION formulations, such as Lumiren® (ferumoxsil), Abdoscan® (ferristene), Feridex® (ferumoxide), Resovist® (ferucarbotran), and Combidex® (ferumoxtran-10), have attained approval for clinical use as MRI contrast agents, allowing the imaging of different tissues and organs (bowel, liver, spleen, lymph node) and thus contributing to the diagnostic of several diseases including cancer, cardiovascular and neurological [18–20]. Combidex® is an ultrasmall SPION (size 20–40 nm) coated with dextran that has been used for lymph node imaging. These SPIONs are able to

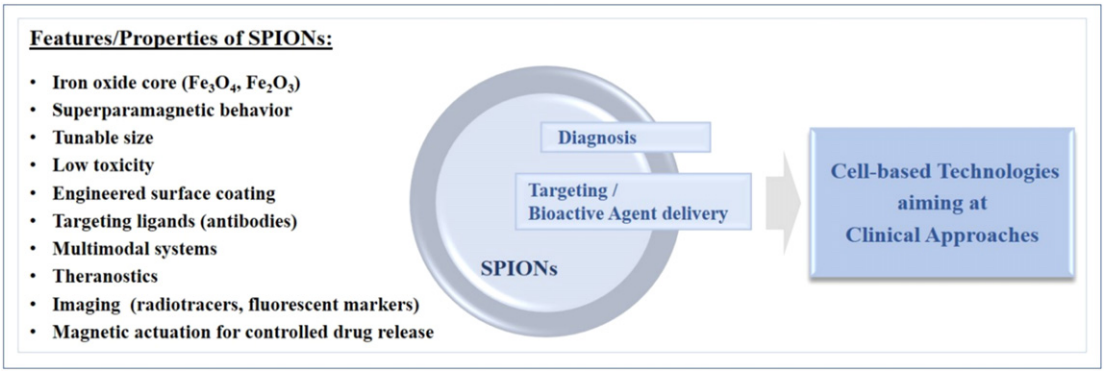

Figure 1. The features of SPIONs and their contribution to cell-based strategies aiming at clinical approaches.

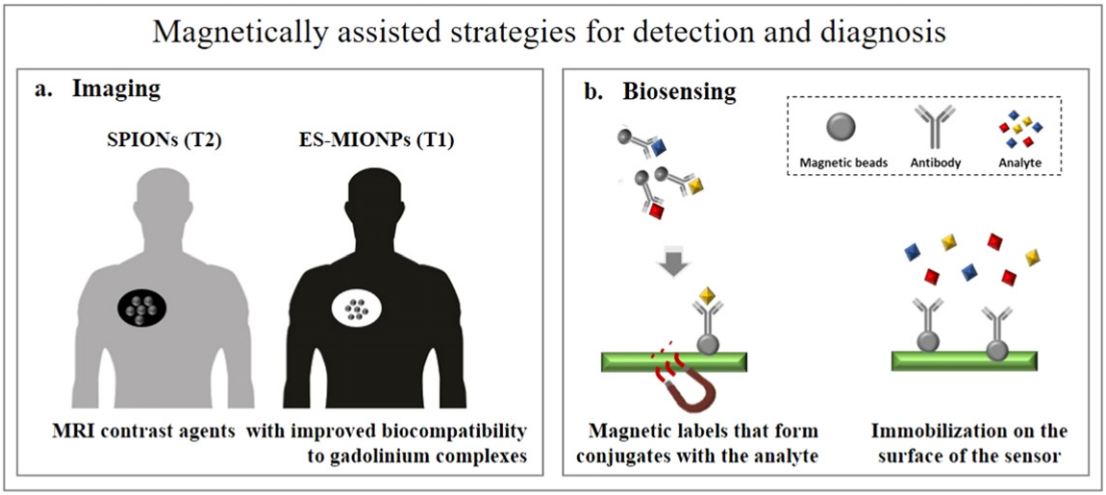

Figure 2. A schematic representation of magnetically assisted strategies for medical diagnosis applications: (a) imaging and (b) biosensing. In MRI imaging (a) SPIONs are T1 contrast agents able to shorten the longitudinal relaxation time, resulting in the brightening (positive contrast) of surrounding tissues, while ES-MIONPs are T2 contrast agents, which are able to shorten the transverse relaxation time resulting in the darkening (negative contrast) of surrounding tissues. Legend: SPIONs—superparamagnetic iron oxide nanoparticles; ES-MIONPs—extremely small magnetic iron oxide nanoparticles.

extravasate from the vasculature to the interstitial space where they are transported to the lymph nodes. The lack of SPIONs in lymph nodes has been associated with the presence of metastases [18]. Another SPION, Feraheme® (ferumoxytol), which was originally developed for iron deficiency treatment in adult patients with chronic kidney disease, is currently under investigation for the detection of inflammatory lesions (clinicaltrials.gov). More recently, extremely small magnetic iron oxide nanoparticles (ES-MIONPs, <5 nm) have been suggested as the probable next generation of MRI contrast agents with the potential to replace Gd-based methods as T1 contrast agents [17, 21]. Along with a better biocompatibility, the hydrodynamic diameter of ES-MIONP is larger than that of Gd-based agents, and consequently, the kidney and liver clearance of ES-MIONPs is slower, thus providing a longer imaging time with higher resolution. Moreover, ES-MIONP clearance from the body (24 h) is still faster than SPIONs [17].

The magnetic labelling of cells, involving the combination of cells with magnetic elements, is an essential *in vitro* procedure and a useful tool to trace and provide information about therapeutic cells *in vivo*, so as to understand the complex role and interactions of implanted cells also concerning safety applications [22–24]. In the work of Swaminathan *et al*, SPION-labelled bone marrow derived from mesenchymal stem cells (BM-SMCs) were guided to the abdominal aortic aneurysm wall through magnetic field actuation, showing that SPION labelling is non-cytotoxic and does not adversely impact the phenotype or elastogenesis by BM-SMCs [24].

More complex approaches have been developed, which highlight SPION technology with the prognostic value in theranostic platforms. Engineering the SPION surface and playing with their properties enables the creation of complex diagnostic units, allowing more efficient detection and treatment action.

With recognition ligands conjugated onto their surfaces, SPIONs can selectively bind to biological entities, including nucleic acids, proteins, viruses, bacteria and eukaryotic cells. Such magnetically labelled targets can be readily distinguished from the remaining sample constituents, because of the intrinsically low magnetic susceptibility of biological objects [25]. Thus, by the magnetization of stem cells that incorporate SPIONs, one might allow for real-time follow-up monitoring by MRI, for example, as a detection tool, but with theranostic revenues, allowing real-time stimulation (mechano-magnetic, drug release) or therapeutic action (drug or gene delivery), and online reports on the engineered system performance (degradation, actuation, temperature or pH levels as an indirect mean to assess local inflammation). The conjugation of ligands on the SPION surface also allows healthy or diseased tissue to be specifically targeted. For example, HER2 is a highly expressed marker on breast and ovarian cancer that combines with magnetoliposomes, forming anti-HER2-magnetoliposome complexes for intelligent MRI [26]. The functionality of this strategy is demonstrated by cellular iron uptake and *ex vivo* imaging [26]. Since patient treatment depends on individual responses to specific biomarkers, targeting imaging may contribute to the detection and prediction of individual patient response and customization of the treatment.

In multimodality imaging approaches, SPIONs are combined with other imaging agents such as fluorescent dyes or radionuclides, which results in an increased amount of complementary information that can benefit more patient-oriented treatment. In order to locate SPIONs within glioma U251 cells, the nanoparticles were functionalized with the tumour-specific ligand folic acid (FA) and also labelled with fluorescein isothiocyanate (FITC) [27]. SPIONs-FA-FITC were effectively internalized by the cells and visualized with MRI, holding promising perspectives for providing dual-modal imaging as non-toxic and target-specific vehicles toward human brain tumour treatment.

SPIONs can also be combined with bioactive molecules with therapeutic value to treat a disease or a pathological condition. Doxorubicin (DOX), a drug used in oncology therapies, was encapsulated in polydopamine-coated SPIONs, which were specifically designed for MRI detection and cell targeting drug delivery [28]. The particles demonstrated a good negative contrast effect for MRI detection, and achieved high efficiency of drug delivery by quick degradation in a reductive environment due to the cleavage of the disulfide linkages. Moreover, the cellular uptake and cytotoxicity of the DOX-loaded SPIONs against HeLa cells, causing their death upon internalization, demonstrated the effectiveness of the developed system towards theranostic strategies.

Since the application of external magnets may limit the targeting of deep tissues or organs as their field strength and field gradient decrease exponentially with distance from the surface, the monitoring or targeting of magnetic labelled systems can be alternatively performed using magnetic resonance targeting. This approach uses the magnetic field gradient coils inherent to all MRI systems, to steer magnetic particles (or the systems containing them) to a target site [29]. Clinically available MRI scanners are able to detect the location of magnetically labelled macrophages carrying an oncolytic virus into primary and metastatic tumour sites, and non-invasively confirm their anatomical position in mice [30].

An emerging imaging technique that is increasingly attracting attention is magnetic particle imaging (MPI). MPI is based on the response of SPIONs to alternating magnetic fields and can specifically determine their spatial distribution and local concentration. Unlike MRI, SPIONs are the only source of the signal, and thus the only visualized elements in MPI. Resovist®, a clinically approved liver MRI contrast agent, is the standard MPI tracer; however, studies on the development of improved tracers to better fit MPI purposes are currently underway [31, 32]. MPI is expected to provide high sensitivity and high spatial resolution [31]. Currently, it is under evaluation in preclinical research studies in vascular imaging, tumour imaging, targeted imaging, transplanted stem cell tracking, and to image medical devices such as guide wires and PTA catheters [33].

2.2. Biosensing

Biosensing is an effective diagnostic platform for the detection of clinically relevant disease biomarkers, pathogens and cells with high sensitivity, which can enable early disease diagnosis and the real-time monitoring of individual responses to treatments. Biosensing strategies focus on monitoring biological events and on the detection and quantification of analytes such as ligands, proteins or other molecules with bioactive action, using a biosensor that converts the biological response into a processable and quantifiable signal [34]. A biosensor typically comprises three elements: (i) a bioreceptor that binds specifically to the analyte; (ii) an interface where a biological event occurs and gives origin to a signal that is picked up by a transducer; and (iii) an electronic system where the transducer signal is converted to an electronic signal and amplified by a detector circuit using an appropriate reference and sent for conversion into a physical parameter describing the biological event under investigation [34].

The application of magnetic nanoparticles (MNPs) in biosensing provides enhanced sensitivity, lower limit of detection, higher signal-to-noise ratio and a shorter analysis time [35, 36]. Moreover, as most biological samples have no magnetic background, unlike other biosensing platforms, highly sensitive measurements can be performed in turbid or otherwise visually obscured samples with minimal processing [37]. MNPs can be immobilized on the surface of the transducer or function as free moving magnetic labels forming analyte-MNP conjugates,

followed by their attraction by an external MF onto the transducer [36, 38].

SPION-based biosensors have been developed using different transduction principles: electrochemical, optical, piezoelectric and magnetic field [36, 37].

An electrochemical cytosensing approach was developed by Zheng *et al* [39] for the selective detection of leukaemia cells and the quantitative evaluation of cell surface receptors DR4 and DR5, which are important diagnostic markers for guiding death-receptor-based leukaemia treatment. The fabricated sensor was based on the co-immobilization of both recombinant human TRAIL—a ligand that specifically binds to DR4 and DR5 causing apoptosis of leukaemia cells—and horseradish peroxidase (HRP) on gold-nanoparticle-decorated magnetic Fe_3O_4 beads. The presented approach achieved a detection limit as low as ~40 cells and allowed the quantitative characterization of the DR4/DR5 expression on leukaemia cells. The developed platform was considered to be of great value for the early diagnosis of human leukaemia and the monitoring of therapeutic effects on leukaemia patients.

Considering that the early detection of cardiac biomarkers such as C-reactive protein (CPR), is essential for cardiovascular diseases, Zhou *et al* [40] developed an ultrasensitive piezoelectric immunosensor based on a capture probe of CPR labelled Fe_3O_4 nanoparticles. The capture probe increased the CRP concentration through magnetic separation, thus amplifying the CPR signal, and also allowing ready immobilization on the electrode by an external magnet. The reported detection limit was 0.3 pg ml^{-1}, which is lower than traditional immunoassay values. The sensor can be regenerated and repeatedly used, representing a versatile technology for the detection of cardiac markers.

SPIONs have also been used to amplify the signal developed by surface plasmon resonance (SPR) spectroscopy in an optical-based biosensor [41]. The experimental results demonstrate that anti-thrombin aptamer-Fe_3O_4 nanoparticle conjugates greatly enhance the sensitivity of the SPR sensor for the detection of thrombin, and hold the potential to be extended to the detection of other biomolecules of interest.

Hathaway *et al* [42] developed a magnetic-field-based biosensor by using a superconducting quantum interference device (SQUID) system for the detection of MCF7/Her2-18 breast cancer cells using an anti-Her2 antibody conjugated to carboxyl-functionalized SPIONs. The authors demonstrated that the system was able to detect as few as 1 million optimally labelled breast cancer cells at a depth of 4.5 cm in a breast phantom model.

Biosensing remains quite an exciting platform for detection and diagnosis. Although currently explored systems relate to single analyte detection, it is likely that biosensing devices will evolve toward multi-analytic detection systems with improved sensitivity and selectivity. Improved systems will also benefit from miniaturization, which will lead to cost reduction in the detection and quantification of biomarkers, thus making them cost effective to compete with commercially available biosensing tools. Future magnetic-based biosensors are also expected to be easily handled without requiring special equipment, and if desired, be integrated into more complex devices such as microfluidic chips. They can also be computer controlled, monitoring real-time events or functional responses in order to study biological mechanisms to be assessed in *in vitro* models.

3. SPIONs as tools for improving cell functionality in cell-based therapies

A growing number of studies show the applicability of magnetic actuators in improved cell separation techniques, receptor-mediated targeting, activation of molecular pathways (via cellular receptor activation), the transport and delivery of gene and pharmacological cargoes and the integration of magnetic responsive systems. These tools with bioinstructive action have been investigated for regenerative medicine and TE approaches (figure 3).

3.1. Immune magnetic sorting/separation

The sorting of biological targets, such as cells or proteins, from heterogeneous pools may result in more efficient usage in specific applications or therapies. The selection of cells from a crude population with a basis on magnetic separation (figure 3(a)) is possible with a recurrence of magnetic elements such as SPIONs, unless the cells possess an intrinsic magnetic moment, which is the case for red blood cells (as the cytoplasm of erythrocytes is rich in hemoglobin, an iron-containing biomolecule), and magnetotactic bacteria (these bacteria have organelles called magnetosomes that contain magnetic crystals).

Immunomagnetic cell isolation relies on the coupling of magnetic particles with antibodies, enabling a cell subpopulation to be selected with putative interest. Briefly, the immunomagnetic beads are conjugated with the antibody of interest and incubated with cells. Subsequently, the bead-bound cells are attracted to the magnet and separated from the unlabelled cells. SPIONs for cell separation should be chemically stable and should not aggregate in the media. Moreover, magnetic particles should also possess low magnetic remanence after having been subjected to the magnetic field and not bind to cells non-specifically. Finally, and anticipating *in vivo* studies, SPIONs for cell isolation should have dimensions which minimize phagocytosis [43].

Previous studies by our research group [44–50] and others [51, 52] show that human adipose stem cells (hASCs) are composed of subpopulations with distinct differentiation potential, which highlights

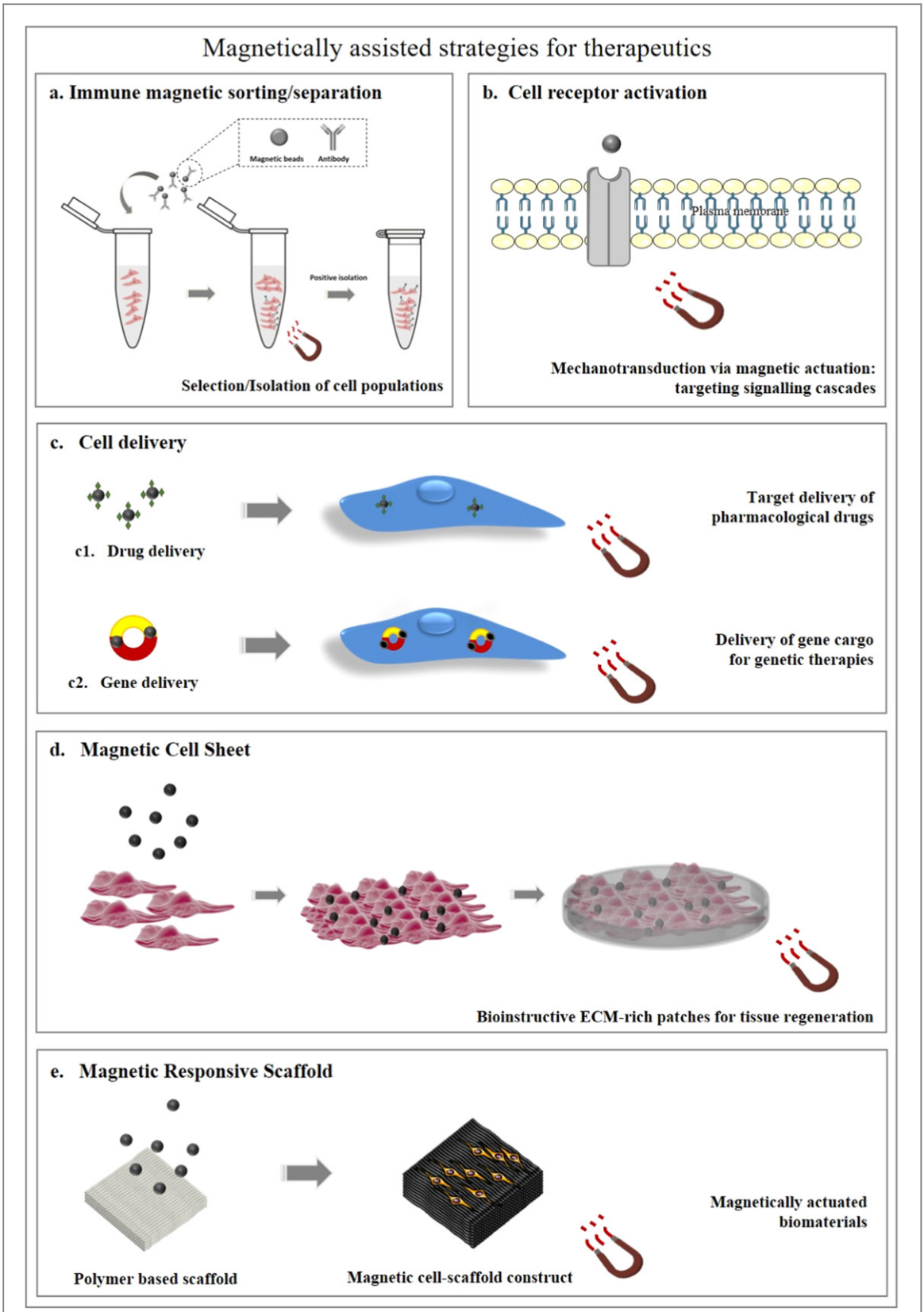

Figure 3. A schematic representation of magnetically assisted cell-based strategies envisioned for therapeutic applications: (a) immune magnetic sorting/separation; (b) magnetic cell receptor activation of mechanotransduction pathways; (c) magnetic bioactive agent delivery: (c1) drug delivery and (c2) gene delivery; (d) magnetic cell sheets; and (e) magnetic responsive scaffolds. Legend: ECM—extracellular matrix.

the differential role of subpopulations to lineage commitment and toward (autologous) tissue-oriented applications. hASCs cell subpopulations have been successfully isolated using commercially available superparamagnetic Dynabeads®, precoated with several antibodies of interest, for instance SSEA-4,

STRO-1 and CD29. These subpopulations demonstrate dissimilar osteogenic and chondrogenic differentiation potential [44–47]. Moreover, the SSEA-4 subpopulation of the cells gives rise to both microvascular endothelial-like and osteoblastic cells [47]. More recently, a subset of hASCs that is positive for tenomodulin (TNMD) expression was investigated for tenogenic lineage commitment [50]. TNMD+ hASCs demonstrated increased tenogenic potential when compared to the crude population.

This is an effective methodology based on magnetic actuated tools for the separation of distinctive stem cell subpopulations within a crude population that might be used with higher efficiency in lineage-specific regeneration therapies. To sum up, the fact that the cells within the heterogeneous pool respond differently and with improved differentiation capability toward crude populations confers an added therapeutic value to cell screening and sorting cell-based strategies in the repair of tissue injuries.

Using the same principle of immunomagnetic separation, the magnetic activated cell sorting (MACS) technique, combines the antibody-SPION labelling of cells with MACS sorter columns by means of a high-gradient magnetic field composed of permanent magnets within the columns. The columns are normally designed for positive selection, but can also be used for the depletion of strongly magnetically labelled cells. Recently, Jia *et al* reported this technique for the purification of MSCs expressing CD90 from synovial fluid (hSF-MSCs) at the knee joint [53]. MSCs have been found to maintain the MSC phenotype, stemness and differentiation potential. However, for *in vitro* diagnostics and in cell-based therapies, it might be desirable to screen multiple targets simultaneously, challenging throughput sorting. Having this in mind, Adams *et al* reported a study using a multi-target magnetic activated cell sorter (MT-MACS) and microfluidics technology to achieve the spatially-addressable sorting of multiple target cell types in a continuous-flow manner [54]. Specifically, two magnetic tags with distinct saturation magnetization and size were used. The hydrodynamic force produced from the laminar flow together with the magnetophoretic force produced by patterned ferromagnetic structures within the microchannel resulted in the selective purification of two types of target cell into multiple independent outlets [54].

MACS offers the advantage of sorting a high number of events/cells, which can be combined with other parameters such as cell size and complexity, allowing the high-throughput separation of magnetically labelled targets. Regardless of the method used, the selection of defined cellular populations will likely induce a more tissue-specific oriented response, leading to cell-based strategies with tissue targeted commitment.

3.2. Activation of mechanotransduction pathways via magnetic actuation

The MNP-mediated activation of signaling pathways may trigger the production of specific biomolecules, such as enzymes or transcription factors, ultimately resulting in altered gene expression, influencing cellular processes such as proliferation, apoptosis, differentiation, inflammation or migration. The activation of signal pathways by MNPs and the understanding of the underlying signalling machinery is increasingly important for knowledge of tissue biology and to predict tissue responses to changes in environmental mechanical stimuli. Therefore, the control of biological responses using MNPs might be desirable in a clinical scenario, which can be synergistically combined with the application of MF actuators, such as magnetotherapy devices, leading to improved outcomes in cell-based therapies.

Unveiling complex mechanisms as the mechanotransduction pathways of cells in musculoskeletal tissues can be achieved by tailoring MNPs with receptor ligands or mechanosensitive binding antibodies (figure 3(b)) [55]. MNPs respond to the application of an oscillating magnetic field, delivering mechanical stimuli directly to individual cells, specifically targeting particular mechanoresponsive cell receptors. MNP technology and the actuation of oscillating magnetic fields have been extensively utilized to remotely activate different signalling pathways, through stimulation of the transmembrane ion channel stretch activated potassium channel (TREK-1), the (integrin) RGD-binding domains [56, 57], the platelet-derived growth factor receptor (PDGFR) [58], and frizzled receptors [59, 60]. These constitute different examples of mechanically responsive targets that have been explored in the osteogenic differentiation of hMSCs. Recently, this biomagnetic technology has been demonstrated in the targeting of the TGF-β/Smad2/3 pathway involved in the mechanotriggering of stem cell tenogenesis [61].

3.3. Target/delivery of bioactive agents

The role of SPIONs in regenerative medicine and disease management can be further extended to the possibility of targeting and delivering bioactive agents, modulating the release profile of the agent and assessing the therapeutic efficacy of a delivered agent in real-time promoting a more efficient treatment.

Bioactive agents such as chemotherapeutic drugs, radionuclides, anti-inflammatory agents or therapeutic peptides/antibodies can be loaded on or within the organic or inorganic surface coating of SPIONs (by a covalent bond or physical interactions), or trapped within complex systems such as magnetoliposomes, microparticulate systems or hydrogel matrices [28, 62–66].

The actuation of an external MF can actively guide the bioactive agents to a specific anatomical site to be

retained (figure 3(c1)), and trigger a remote-controlled release that can be modulated according to the treatment requirements [67, 68].

Static or low frequency MFs trigger bioactive agent release by mechanical disruption of the magnetic carrier, with negligible or non-existent temperature effects [66, 67, 69]. The application of an oscillating MF (33 Hz, 0.18 T) in alginate/chitosan beads containing SPIONs and insulin prepared by Finotelli *et al* [69] increased insulin release three-fold relative to a no-field control. This was a promising result envisioning the clinical applications of the controlled release of insulin following a subcutaneous implant approach. SPION incorporation into hydrogel matrices has also been explored as a delivery system. Hydrogels contain a large amount of water, which gives them physical similarity to tissue, high biocompatibility and the ability to encapsulate hydrophilic bioactive agents and even cells. Static magnetic fields (magnets) can deform the hydrogels containing the SPION (ferrogels) network resulting in controlled drug (mitoxantrone), biomacromolecules such as DNA and cell release [70, 71].

Studies by Campbell *et al* [72] suggest the combination of thermal responsive microgels and SPIONs to enhance bioactive agent release by the actuation of a high-frequency MF that increases the temperature locally. SPIONs containing hydrogels obtained by copolymerizing N-isopropylacrylamide (NIPAM) with N-isopropylmethacrylamide (NIPMAM) enhanced the release of FITC-dextran four-fold, and was used as a therapeutic model for tracking release, when exposed to an alternating magnetic field (200 kHz) relative to the off state [72]. The actuation of the alternating magnetic field induced an increment in temperature from 37 °C to 43 °C. Moreover, a pulsatile release was externally controlled over multiple cycles and multiple days, suggesting system applicability for treatments that would benefit from repeated, pulsatile drug release [72].

Thus, SPION technologies can reduce the therapeutic dosages administered, reduce the side effects caused by higher dosages in systemic circulation, decrease (undesired) diffusion into surrounding zones and improve treatment efficiency. Moreover, magnetic carriers developed for bioactive agent delivery can retain their ability to be imaged by MRI. Thus, the diseased tissue can first be located by conventional MRI techniques and the incorporated agent monitored and released as required (image guided therapy) [73].

The potential of SPION systems for targeted and controlled drug delivery has mainly been employed for chemotherapeutic and radiotherapeutic delivery for cancer treatment having reached the clinical stage [74]. But the delivery of other drugs/biomolecules in SPION delivery systems for diseases such as heart/cardiac diseases [75], brain disorders [76, 77] are also under study.

Furthermore, there is the possibility of incorporating multiple biomolecules with joined or sequential release to modulate the particular cell response of a given tissue. One example takes advantage of this approach to study inflamed tissue and control inflammation stages, which may be associated with the mechanisms of disease.

The release of agents with biological relevance can also contribute to the establishment of models to predict individual behaviours for a regimen involving a single drug or a cocktail of drugs, scaling up the most promising agents to clinics, and personalizing strategies to hinder the progression of disease and envision an optimized treatment for each patient.

3.4. Magnetofection and gene delivery

Magnetofection is defined as the delivery of nucleic acids, either 'naked' or packaged (as complexes with lipids or polymers, and viruses) into the target cells using MNPs under the guidance of an external MF [78]. The advantage of magnetofection over other transfection methods is its high efficiency, short-term incubation protocols, low toxicity, and the avoidance of using viral vectors, which are associated with adverse immunogenic responses and mutagenesis [79, 80].

The cationic polymer polyethylenimine (PEI) is the gold standard for nucleic acid magnetofection; however, it exhibits a well-known dose-dependent cytotoxicity [81]. SPIONs modified with cationic lipids or polymers can bind electrostatically to a negatively charged phosphate backbone of genetic material, forming magnetic complexes to be transfected into cells under the application of an MF [79, 82], integrating the genetic material of the cell, upregulating desirable genes and leading to the synthesis of therapeutic proteins. An effective gene delivery and imaging system to target hepatocytes has been developed by Cheong *et al* [83] to help in the diagnosis and treatment of liver diseases. SPIONs were loaded into chitosan-conjugated linoleic acid nanoparticles and further associated with a plasmid vector containing the enhanced green fluorescence protein (pEGFP). Linoleic acid was used to target the hepatocytes and chitosan to complex with the plasmid vector. The GFP expression was detected in the cytoplasm of hepatocytes *in vitro*, and after an intravenous injection in mice it was possible to monitor the selective accumulation of the nanoparticles in hepatocytes by MRI, allowing gene mobility to be tracked.

Besides gene therapies, SPION technology can be used in guiding cell processes such as lineage differentiation (figure 3(c2)).

An interesting combined approach is 'magselectofection', in which genetic modification and cell isolation are integrated in a single procedure proposed by Sanchez-Antequera *et al* [84], combining magnetic non-viral and viral vector compositions and MACS columns. This technique was recently employed in the application of magnetic cell sorting after hMSC transfection with magnetic capsules (intracellular delivery of messenger RNA). The magnetic sorting process

allowed both hMSCs containing magnetic capsules and hMSCs without magnetic capsules to be obtained [85].

Successful cell programming with SPIONs is envisioned to contribute to efficient cell differentiation protocols, establish proper *in vitro* models to better understand the molecular mechanisms underlying regeneration and assist the development of improved regenerative medicine strategies.

3.5. Magnetite-functionalized cells (cell sheets)

The integration of cell biology within nanomaterials science results in highly specific approaches that can result in smart responsive cell sheets, a new generation of cell sheet technologies, as tissue substitutes. The rationale of this approach is based on the fact that cells synthesize their own tissue-specific extracellular matrix (ECM) following structural and hierarchical coordination, bypassing the need for artificial devices and their shortfalls [86].

First termed 'magnetic force-based tissue engineering' (Mag-TE), by Akira Ito and co-workers in 2004 [87], this concept names a construction technique of magnetically labelled cell sheets, where magnetic force is applied to manipulate the attachment and release of (figure 3(d)) an integrative and stable extracellular rich layer of cells. Magnetic scaffold-free constructs are suggested as cohesive tissue-like matrices for tissue augmentation capable of magnetic responsiveness and guidance upon implantation. In the study proposed by Ito and co-authors [87], magnetite cationic liposomes were taken up by keratinocytes to magnetically label the cells. The labelled cells were then seeded in ultralow-attachment plates under a vertical magnetic force provided by a neodymium magnet. One day after, the magnet was removed and the magnetic keratinocyte sheets were harvested using a cylindrical alnico magnet.

In another work performed by Shimizu and colleagues, this 'Mag-TE' technique was used to transplant mesenchymal stem cell (MSC) sheets into a cranial bone defect in nude rats using an electromagnet [88], resulting in new bone formation surrounded by osteoblast-like cells in the area of the defect. Ishii *et al* [89] also investigated the therapeutic potential of magnetic MSC sheets for reparative angiogenesis in a mouse model of hind limb ischemia. Animals treated with magnetized MSC sheets placed on top of the ischemic adductor muscles showed a greater degree of blood perfusion, increased expression of vascular endothelial growth factor (VEGF) and reduced apoptosis in ischemic tissues [89] in comparison to animals treated with injected magnetized MSCs. Similarly, magnetite cationic liposomes (MCL) were used to magnetically label C2C12 myoblast cells for the construction of three-dimensional artificial skeletal muscle tissues by magnetic force [90, 91]. Herein, MCL-labelled C2C12 cells were seeded into the gap between the well wall and a polycarbonate cylinder, and a magnet was placed under the well [91]. These ring-shaped cellular constructs were subsequently removed and hooked around two stainless-steel minutien pins. The fabricated constructs expressed myogenic markers during differentiation and contracted to generate physical forces in response to electrical stimulation.

More recently, Kito and colleagues investigated the therapeutic potential of combining induced pluripotent stem (iPS) technology with magnetic cell sheets for reparative angiogenesis [92]. iPS cells generated from mouse skin fibroblasts were induced in iPS cell-derived fetal liver kinase-1 positive (Flk-1^{+}), incubated with MCLs and mixed with a diluted ECM precursor embedding system (type I collagen and matrigel). Then, a magnet was placed on the reverse side in order to form a multi-layered cell sheet. The implantation of the magnetized iPS cell sheet accelerated the revascularization of the ischemic hindlimbs relative to the contralateral limbs in nude mice, with increased expression of VEGF and bFGF in the ischemic tissue [92].

In these studies, the potential to use magnetically functionalized cells from different sources to construct multi-layered cell sheets has been demonstrated for different applications as musculoskeletal tissue regeneration and vascularization using permanent magnets that, by definition, generate static magnetic fields which do not vary over time. However, the properties of a magnetic field, such as strength/intensity and exposure time, may result in a mechano-stimulus effect at the cellular level. This effect is particularly interesting for studies of musculoskeletal tissues and may contribute to the understanding of mechanoresponsive processes in cells using magnetic force-based tissue engineering. Gonçalves *et al* proposed a biocompatible and bioinstructive extracellular-rich patch made of a magnetically labelled cell sheet of a subset of stem cells isolated from adipose tissue with improved tenogenic capability, for the regeneration of tenotopic defects [93]. The developed cell sheet exhibited a tendon-like ECM, demonstrating good mechanoelastic properties and magnetic responsiveness capability, thus suggesting the applicability of these scaffold free patches as augmentation tissue substitutes [93].

3.6. Magnetic responsive systems for tissue engineering strategies

Strategies focusing on the development of magnetically actuated biomaterials have been investigated for tendon, bone, osteochondral, muscle, cardiac TE and vascularization (table 1).

The rationale for using 3D architectures doped with magnetic nanoparticles relies on the provision of small stimulation point constituents of the scaffold's polymer/hydrogel matrix. This allows the scaffold to

Table 1. Magnetic scaffolds on TE approaches, from 2010–2017.

Scaffold base material(s)	Magnetic component(s)	Magnetization value (emu g^{-1})	Final application	Reference
Hydroxyapatite (HA) and collagen	Ferrofluids of magnetite nanoparticles coated with: dextransulfate and functionalized with sodium sulfate functional groups (R-OSO_3^-, Na^+); poly-DL-aspartic acid and functionalized with sodium carboxylate (-COO^-, Na^+); starch and functionalized with phosphate groups.	0.14–15	Bone TE	[94]
Poly (D,L-lactide) (PLA) and hydroxyapatite (HA)	Nanoparticles of γ-Fe_2O_3 coated with meso-2, 3-dimercaptosuccinic acid (DMSA)	0.0492	Bone TE	[95]
Poly(ε-caprolactone) (PCL)	Polyvinylpyrrolidone (PVP) coated Fe_3O_4 nanoparticles	3–6	Bone TE	[96]
Poly(ε-caprolactone) (PCL)	Superparamagnetic iron-doped hydroxyapatite (FeHA) nanoparticles	0.1–0.3	Bone TE	[97]
Alginate	Magnetite obtained from ferric chloride hexahydrate and ferrous chloride tetrahydrate	24	Vascularization	[98]
Hydroxyapatite (HA)	Magnetite powders	34 and 53	Bone TE	[99]
Hydroxyapatite (HA)	Magnetic nanoparticles colloids	0.24–0.94	Bone TE	[100]
Poly(lactic-co-glycolic acid) (PLGA)	Superparamagnetic magnetite nanoparticles	3.57–10.01	Bone TE	[101]
Poly(ε-caprolactone) (PCL) and hydroxyapatite (HA)	Iron-doped hydroxyapatite (FeHA) nanoparticles	0.2–1	Bone TE	[102]
Poly(lactic-co-glycolide) (PLGA)	Magnetic nanoparticles	0.54–5.46	Aligned TE	[103]
Poly(ε-caprolactone) (PCL)	Magnetic nanoparticles	1–11.2	Bone TE	[104]
Gelatin–siloxane	Magnetic nanoparticles	0.24–0.64	Bone TE	[105]
Starch and polycaprolactone (SPCL)	Iron oxide nanoparticles	1.22	Tendon TE	[106]
Poly(ε-caprolactone) (PCL)	Iron(III) acetylacetonate	1.7 and 4.8	Bone TE	[107]
Poly(ε-caprolactone) and poly(ethylene glycol)	Magnetic nanoparticles	—	Osteochondral TE	[108]
Poly(ε-caprolactone) (PCL)	Iron oxide (Fe_3O_4)	0.2–1	Bone TE	[109]
Nano-hydroxyapatite (n-HA) and L-polylactic acid (PLLA)	Magnetic nanoparticles (Fe_2O_3)	—	Bone TE	[110]

respond to the application of an external magnetic field, by means of its stimulating intrinsic components [111]. It is thus undoubtedly advantageous to have this additional scaffold function when it comes to providing local and controlled stimulation at the site of injury, exploiting magnetic forces to improve implant fixation, and control tissue morphology along regeneration.

The creation of a polymeric magnetic aligned scaffold using the rapid prototyping technique (figure 3(e)) was described by Gonçalves *et al* [106] for tendon tissue engineering combining a blend of starch, polycaprolactone and iron oxide MNPs. hASCs cultured onto the aligned magnetic scaffolds resulted in the synthesis of a tenascin C and collagen type I rich matrix in magneto-stimulation conditions.

Yulia Sapir [98] also reported the development of alginate scaffolds impregnated with magnetite, which when combined with an alternating field promoted the organization of endothelial cells into capillary-like structures *in vitro*. The same author further studied the response of cardiac cells seeded onto these scaffolds [112]. Short-term stimulation of the cardiac cells in an MNP-impregnated scaffold induced AKT activation by a factor of 1.8. Moreover, a three-fold greater metabolic activity and increased protein levels of troponin-T and connexin-43 were observed in stimulated cardiac cell constructs compared to the non-stimulated ones.

Despite the wide range of applications investigated, magnetic elements incorporated within 3D matrices seem to improve the biofunctionality of the tissue substitutes for TE applications [113]. Besides the magnetic responsive polymer-based scaffolds, hydrogel matrices incorporating MNPs have also been developed for musculoskeletal tissue engineering [114, 115]. A photocrosslinkable magnetic responsive hydrogel made of methacrylated chondroitin sulfate (MA-CS) enriched with platelet lysate was reported to modulate the growth factor release and swelling, as well as having an impact on both the cell morphology and the expression and synthesis of the tendon- and bone-like matrix [114]. Cezar *et al* also reported the remote magnetic actuation of biphasic ferrogel scaffolds composed of alginate with 7 wt% iron oxide implanted in a mouse muscle model [116]. The external magnetic field resulted in uniform cyclic compressions that led to reduced fibrous capsule formation and reduced fibrosis and inflammation in the injured muscle. Moreover, ferrogel-driven mechanical compressions led to enhanced muscle regeneration and a ~threefold increase in the maximum contractile force of the treated muscle at two weeks compared with the no-treatment controls.

With the advances of nanoplatforms in recent years, SPION technology and magnetic actuated systems have arisen with elegant solutions for diagnosis and attractive possibilities for regenerative strategies and therapeutic approaches. Despite the promising results and safety studies, some of the proposed approaches require further validation before becoming available to the clinical field. With the exception of SPIONs and ES-MIONPs used as MRI contrast agents, the magnetic tools proposed for both diagnosis and therapeutics are limited to research and development.

The cell-based studies performed with different cell sources and soluble agents, including drugs and bioactive molecules in magnetic actuated systems for applications as diverse as cell tracking, monitoring or promoting cellular mechanisms as proliferation, differentiation and tissue regeneration justify the continuous improvement of these strategies and demand translational development into clinics.

4. Future perspectives

It is anticipated that the growing investigation of magnetic actuated strategies in the biomedical field and their promising results will have an impact on human healthcare, from cell tracking and imaging techniques to gene and drug screening, and contribute to the identification of biomolecular mechanisms and the establishment of biological timelines in homeostasis and the progression of disease.

The versatility of SPION-based technologies in isolating, screening and characterizing cell populations of therapeutic interest is encouraging the research of new media for cell differentiation as well as the identification and characterization of cell subsets within heterogeneous cellular pools. The availability of cells with enhanced therapeutic value will likely reduce the number of cells required for cell therapies and the costs associated with isolation and culturing methodologies *in vitro*.

Cells naturally respond to magnetic forces and to the magnetic elements used for cell labelling. Since magnetic parameters can be accurately controlled and manipulated, the stimulation, maturation and functionality of implantable cells with or without sophisticated carriers and cargoes (such as genes or drugs) enables the combinatorial effects and more predictive outcomes of cell behaviour to a particular environment, including non-physiological/pathological conditions. SPION-based technologies allow online monitoring upon implantation, enabling a non-invasive and real-time follow-up, providing information on the success of a therapy and the welfare of the patient. Thereupon, with this dual imaging/therapy approach, treatment can be rapidly adjusted, if needed, manipulating the external magnetic field, with minimal discomfort or potential side effects.

Thus, SPION technologies will also advance the field of tissue engineering and regenerative medicine (TERM), more specifically magTERM, contributing to the establishment of labelling and differentiation protocols, to the optimization of novel tools for gene or protein screening and delivery, to the manipulation of

therapeutic cells in the presence or absence of sophisticated matrices, which ultimately sustain repair and/or regeneration, and enable remote controlled action over tissue-engineered constructs *in vitro* and *in vivo*. Therefore, strategies combining magnetically actuated cells and/or magnetic responsive biomaterials would lead to smartly instructive systems fully committed to tissue substitution and regeneration, bolstering the progress of regenerative medicine strategies.

Magnetic actuated systems represent major advances in the fields of nanomedicine and personalized medicine, able to combine diagnostic agents and therapeutic cues as powerful theranostic platforms of predictive action for clinical applications. The successful achievements of such sophisticated tools with biological significance and personalized implications for tissue healing and regeneration will pave the way for the translation of novel biomedical technologies and will assist the healthcare system with more sophisticated and innovative therapies, contributing to the quality of life of patients worldwide.

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