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Review

Use of nanoparticles in skeletal tissue regeneration and engineering

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Summary. Bone and osteochondral defects represent one of the major causes of disabilities in the world. Derived from traumas and degenerative pathologies, these lesions cause severe pain, joint deformity, and loss of joint motion. The standard treatments in clinical practice present several limitations. By producing functional substitutes for damaged tissues, tissue engineering has emerged as an alternative in the treatment of defects in the skeletal system. Despite promising preliminary clinical outcomes, several limitations remain. Nanotechnologies could offer new solutions to overcome those limitations, generating materials more closely mimicking the structures present in naturally occurring systems. Nanostructures comparable in size to those appearing in natural bone and cartilage have thus become relevant in skeletal tissue engineering. In particular, nanoparticles allow for a unique combination of approaches (e.g. cell labelling, scaffold modification or drug and gene delivery) inside single integrated systems for optimized tissue regeneration. In the present review, the main types of nanoparticles and the current strategies for their application to skeletal tissue engineering are described. The collection of studies herein considered confirms that advanced nanomaterials will be determinant in the design of regenerative therapeutic protocols for skeletal lesions in the future.

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Abbreviations. ACs, Articular chondrocytes; ADSCs, Adipose-derived stem cells; AgNPs, Silver NPs; Akt, Protein kinase B; ALP, Alkaline phosphatase; AMF, Alternated magnetic fields; AuNPs, Gold NPs; BCP, Biphasic calcium phosphate; BGNs, Bioactive glass nanoparticles; BMP, Bone morphogenetic protein; BMSCs, Bone marrow stromal cells; BSA, Bovine serum albumin; BTE, Bone tissue engineering; CBD, Collagen-binding domain; CNFs, Carbon nanofibers; CNTs, Carbon nanotubes; CQDs, Carbon quantum dots; CTE, Cartilage tissue engineering; DMX, Dexamethasone; DWCNTs, Double-walled carbon nanotubes; ECM, Extracellular matrix; FeHA, Iron-doped hydroxyapatite; g-CSF, Granulocyte colony-stimulating factor; GAGs, Glycosaminoglycans; GNPs, Graphene nanoplatelets; GONPs, Graphene oxide nanoplatelets; HA, Hydroxyapatite; HUVECs, Human umbilical vein endothelial cells; IGF, Insulin growth factor; IONPs, Iron oxide NPs; IPFP, Infrapatellar fat pad; LBNPs, Lipid-based nanoparticles; MMP, Matrix metalloproteinases; MRI, Magnetic resonance imaging; MSCs, Mesenchymal stem cells; MSNPs, Molybdenum disulfide nanoplatelets; MWCNTs, Multi-walled carbon nanotubes; NDs, Nanodiamonds; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; nHA, Nano-hydroxyapatite; NMs, Nanomaterials; NPs, Nanoparticles; OA, Osteoarthritis; PA, Polyamine; PBMC, Peripheral blood mononuclear cells; PCL, Poly(ε-caprolactone); PE, Polyethylene; PLGA, Poly (lactic-co-glycolic acid); PNIPAM, Poly(N isopropylacrylamide); PTH, Parathyroid hormone; PU, Polyurethane; QDs, Quantum dots; RANKL, Receptor activator of nuclear factor-κB ligand; Runx2, Run-t related transcription factor; SPIONs, Superparamagnetic iron oxide nanoparticles; SWCNTs, Single-walled carbon nanotubes; TE, Tissue engineering; TGFβ, Transforming growth factor beta; TLR, Toll-like receptor; TNF, Tumor necrosis factor; TRAP, Tartrate-resistant acid phosphatase; VEGF, Vascular endothelial growth factor; WSNTs, Tungsten disulfide nanotubes.

Introduction

Nanoscale materials and devices are already being highly researched in science and engineering. Their design, fabrication, and functionality are the focus of the multidisciplinary field of nanotechnology, raising great expectations for several medical challenges (Kestell and DeLorey, 2009). Many efforts are dedicated to the development of novel, specific and selective medical products intended for therapeutic purposes including drug delivery and cell therapy, tissue regeneration, imaging and diagnosis, etc. (Brigger et al., 2002; Koo et al., 2005; Wang et al., 2008; Kulshrestha and Khan, 2018). Nanotechnological tools called nanoparticles (NPs) are particulate materials on a scale between 1 to 100 nm in at least one dimension (Kestell and DeLorey, 2009). NPs are therefore dimensionally comparable to biomolecules such as proteins (1-20 nm), DNA (with a diameter of ~2 nm), hemoglobin (~5 nm), viruses (~20 nm) or cell membranes (~6-10 nm). Thanks to their small size but high surface to volume ratio, NPs diffuse across cell membranes more easily, retain superior potential for controlled drug release and site-specific drug targeting, and superior suitability for intravenous administration than larger microparticles (Shang et al., 2014). However, in addition to intravenous injection, they can also be delivered through many other routes such as the respiratory, dermal and digestive absorption. Once at the target organ, they accomplish their biological effects, mainly consisting of regulation of cell functions, such as modulation of intracellular molecular pathways, apoptosis, oxidative stress, and DNA damage (Panyam and Labhasetwar, 2003; Shi et al., 2010). In fact, NPs can provide targeted, controlled and sustained therapeutic activity to almost any organ/area of the body when used as a drug delivery system, since they can load a large variety of medications. Hydrophilic and hydrophobic drugs, and other bioactive molecules (proteins or peptides, macromolecules, antibodies, plasmids and vaccines) can be trapped, encapsulated, adsorbed, attached or dissolved on and into NPs (Choi and Han, 2017). Moreover, nanotechnologies are expected to advance other applicative fields of biomedicine like regenerative medicine, a tissue regeneration technique aimed at restoring the function of lost, damaged, or aging cells in order to replace and repair diseased tissue and organs (Mason and Dunnill, 2008). The social impact of this research is extremely relevant since it will allow for implantation of natural, synthetic, or semi-synthetic tissues and organs, either fully functional from the beginning, or growing into the required functionality (Ciccocioppo et al., 2019). The generation of new tissues and organs involves cells and scaffolds, namely three-dimensional (3D) structures able to host cells during their proliferation and differentiation into different cell types (Pina et al., 2019). The manipulation of engineered materials (e.g. by introducing growth factors) is often needed to recapitulate biological growth and regenerative processes and produce fully functional tissues suitable for implantation (Williams, 2019). However, even though sophisticated, such strategies often fail to faithfully mimic the complex properties of natural tissues (Williams, 2019). Recently, innovative solutions offered by nanotechnologies were proposed to address this challenge by improving mechanical and biological performances of tissue-mimicking materials. Among numerous functions, NPs can serve for improvement of: (i) biological properties of cells and molecular modulation of their behaviour (Parizek et al., 2012; Ghasemi-Mobarakeh et al., 2015), (ii) mechanical or electrical properties of scaffolds and other supporting materials (Fleischer et al., 2014; Corona-Gomez et al., 2016), (iii) antibacterial activity (Ignjatović et al., 2016; Reshma et al., 2017), (iv) local delivery of bioactive molecules (Vo et al., 2012; Liu et al., 2015; Zhao et al., 2015), (v) remote control of cell position (patterning) and generation of oriented engineered structures (Sensenig et al., 2013). Techniques of bio-sensing, molecular detection, gene delivery, DNA transfection, viral transduction and remote guidance of cells can be mediated by nano-objects in order to facilitate the growth of different types of tissues (Van Rijt and Habibovic, 2017). Silver, metal oxides and other metals are popular materials for the preparation of particulate materials dedicated to *in situ* tissue implantation thanks to their marked antimicrobial activity (Patil and Singh, 2019). Moreover, certain other metals have additional favourable properties such as the versatility of surface conjugation and electrical conductivity of gold and the magnetic responsiveness of iron. Particularly, NPs endowed with magnetism are used for gene and drug delivery, for stimulation of cell pathways including those related to mechanotransduction and for imaging and guiding the cell patterning in the generation of complex 3D tissues (Sensenig et al., 2013; Van Rijt and Habibovic, 2017). The stability and brightness of luminescent quantum dots (QDs) is helpful for optical bio-sensing (Zhao et al., 2017). The stimulation of unique electromechanical properties makes carbon nanotubes (CNTs) attractive tools to electrochemically detect biological events (Farrera et al., 2017; Raphey et al., 2019).

By virtue of their high biocompatibility, low immunogenicity, and their intrinsic properties, these objects hold great promise to overcome the technical limitations in material engineering, addressing the current hurdles in TE. Here, we present an overview of various types of NPs (magnetic, metallic, organic, ceramic and metal oxide NPs) and their potential in regenerative medicine, aiming at giving a comprehensive vision of the field and at inspiring tissue engineers by guiding them through the plethora of NP applications, in order to help them find the one that best fits their needs.

Nanoparticles

Various materials, nano-scaled in at least one dimension can be produced. Conventional nanoparticles and quantum dots are examples of three-dimensionally nanosized objects, but nanoscale can also be applied to one single (such as in very thin layers or surface coatings) or two dimensions (such as nanotubes and nanowires) (Pokropivny and Skorokhod, 2007; Jeevanandam et al., 2018). More specifically, a nanoparticle is a generic nano-object with three external nanoscale dimensions. The terms nanoplate and nanorod are instead used when at least one or two dimensions are in the nanoscale, whereas the other dimensions are usually larger. The bulk properties of any material merely result from the superposition of all the quantum forces, affecting all the atoms composing the material. Reducing the dimensions to nanoscale can thus break a size barrier below which averaging cannot depict a realistic picture anymore, so the study of specific behaviour of individual atoms or molecules becomes crucial. In this way, nanomaterials (NMs) may drastically change their characteristics from their counterpart seen on the macroscale level (Jeevanandam et al., 2018): for instance, solidity at room temperature turns into liquid state in nano-gold (Panahi et al., 2017), and the optical opacity of copper becomes transparency (Nam and Lee, 2016). Other metals from stable become combustible (aluminium) (Martin et al., 2018), or change from chemically inert to catalysts (platinum) (Kadirgan et al., 2009; Chaturvedia et al., 2012). Insulators like silicon acquire conductive capacity (Zhang and Lieber, 2016).

These effects maximize in the particulate matter with reducing size. In NPs, both a bulk nature and molecular properties converge, making them behave as powdered-like ensemble and agglomeration of singular entities at the same time (Chekman, 2013). In fact, whereas the properties of the purely bulky substances are mainly dependent on the internal atoms which represent the predominant component, in NPs the contribution from the surface atoms are important. The surface atoms experience the external environment and sense different conditions with respect to the internal ones, displaying therefore different reactions to stimuli. With great reduction of particle size, the quantization of energy for the electrons in solids becomes dominant (quantum size effect), being instead not relevant in micro and macro-materials (Khang et al., 2007; Li et al., 2014). The magnetic, electric and optical behaviours of nanomatter can be strongly affected by the quantum effect. In the perspective of orchestrating cell-NP interactions, this phenomenon can be exploited to have strict control over the surface energy in order to regulate initial protein adsorption (Khang et al., 2007). Like the quantum size, the small size effect and surface effect have a direct influence on the physico-chemistry of nanostructured objects, their fate and behaviour in living systems (Li et al., 2014). If compared to the same mass in bulk form, nanoscaled substances have a larger surface area, affecting their physical or electrical attributes, and enhancing or activating their chemical reactivity (Khan et al., 2017). The high surface to volume ratio, the high mobility in a free state and the consequent slow sedimentation rate provide advantages in terms of manipulation, modification and applicative use of these materials (Khan et al., 2017).

NPs made of carbon, metal, ceramic, polymers, semiconductors or lipids (Fig. 1), produced and characterized through different techniques (Jain and Thareja, 2019; Modena et al., 2019), are commonly used in a variety of research fields, e.g., biomedical sciences, electronics, optics and more.

Carbon-based NPs

The single-atom-thick planar sheet of sp2-bonded carbon atoms arranged in a hexagonal or honeycomb lattice, named graphene, served as basic supra-atomic element for the development of other morphological variations, evolving into hollow tubes, spheres, and ellipsoids (Bakry et al., 2007; Nasir et al., 2018). Several layers of graphene make up the graphite and diamond structures, when containing two and three dimensional lattice bonds, respectively. Other three dimensional carbon-based NPs are represented by the fullerene family, the most known variation being C60, also called "Buckyball". Manipulation of surface chemistry has created diverse populations of water-soluble derivatives of fullerenes with different behaviours (Bakry et al., 2007; Ruiz et al., 2014; Moreira et al., 2015). The winding of graphene results in cylindrical nanostructures known as Carbon nanotubes (CNTs) (Bogdanović and Djordjević, 2016), that can be divided into three types: single-walled carbon nanotubes (SWCNTs), double-walled (DWCNT), and multi-walled carbon nanotubes (MWCNTs). Other derived materials account for fluorescent Carbon Quantum Dots (CQDs), carbon nanofibers (CNFs), nanodiamonds (NDs) and graphene oxides. Based on their shapes, carbon-based NPs are categorized in (i) zero dimensional or spherical (such as NDs), one-dimensional or cylindrical (such as CNTs), and (iii) two-dimensional NPs (such as graphene nanoplatelets, GNPs). Due to their high stability, purely carbon-based materials have already been extensively used in the medical field (Bogdanović and Djordjević, 2016), and also in a variety of other different fields (Jariwala et al., 2013).

Inorganic NPs

Inorganic NPs include metal, metal oxide and semiconductor-made NPs. Metals frequently involved are iron, gold, silver, manganese, titanium, copper, zinc, zirconium, cobalt, nickel, tungsten, and vanadium (George et al., 2018). Due to the ease of surface functionalization, chemo-physical stability and tuneable optical characteristics, in the last decade there has been

significant research on gold NPs (Au-NPs) and their biomedical applications, especially in the area of controllable drug release. In parallel, 'supermagnetism' has made of iron oxide NPs (IONPs) successful effectors of targeted drug delivery, especially in the context of tumor treatment, since after drug-loading their accumulation at the tumor site can be remotely induced by an external magnetic field (Ali et al., 2016). Surface functionalization with targeting vectors and magnetic guidance are popular strategies to perform targeted therapy to kill cancer cells without harming healthy cells (Wu et al., 2008; Jahangirian et al., 2019). IONPs also present intrinsic hyperthermia and photo-thermal abilities important for localized cancer treatment (Estelrich and Busquets, 2018). Both Au-NPs and IONPs are also contrast agents, therefore their distribution in tumors and other organs can be imaged (Ali et al., 2016; Bordeianu et al., 2018). Metal and metal oxide NPs have been extensively investigated in respect to their antimicrobial and antibacterial effects as well (Dizaj et al., 2014). For instance, besides exerting anti-angiogenic, anti-inflammatory and anti-cancer activity, silver NPs (AgNPs) have been recognized to act as multifunctional antagonists of bacteria, fungi, and viruses (Abbasi et al., 2016; Zhang et al., 2016; Qing et al., 2018). Attention has also been paid to manganese oxides (Mn-oxides), which can be found in various forms like MnO, Mn_5O_8, Mn_2O_3, MnO_2, and Mn_3O_4, emerging as promising candidates for high-performance and sustainable applications in catalysis, biomedicine (Chevallier et al., 2014), biosensors, electrochemistry, photoelectronics and electronics (Hoseinpour and Ghaemi, 2018). Semiconductors such as silicon and ceramics can generate NPs as well. Primarily made up of oxides, phosphates, carbides, and carbonates of metals and metalloids (such as calcium, titanium, silicon),

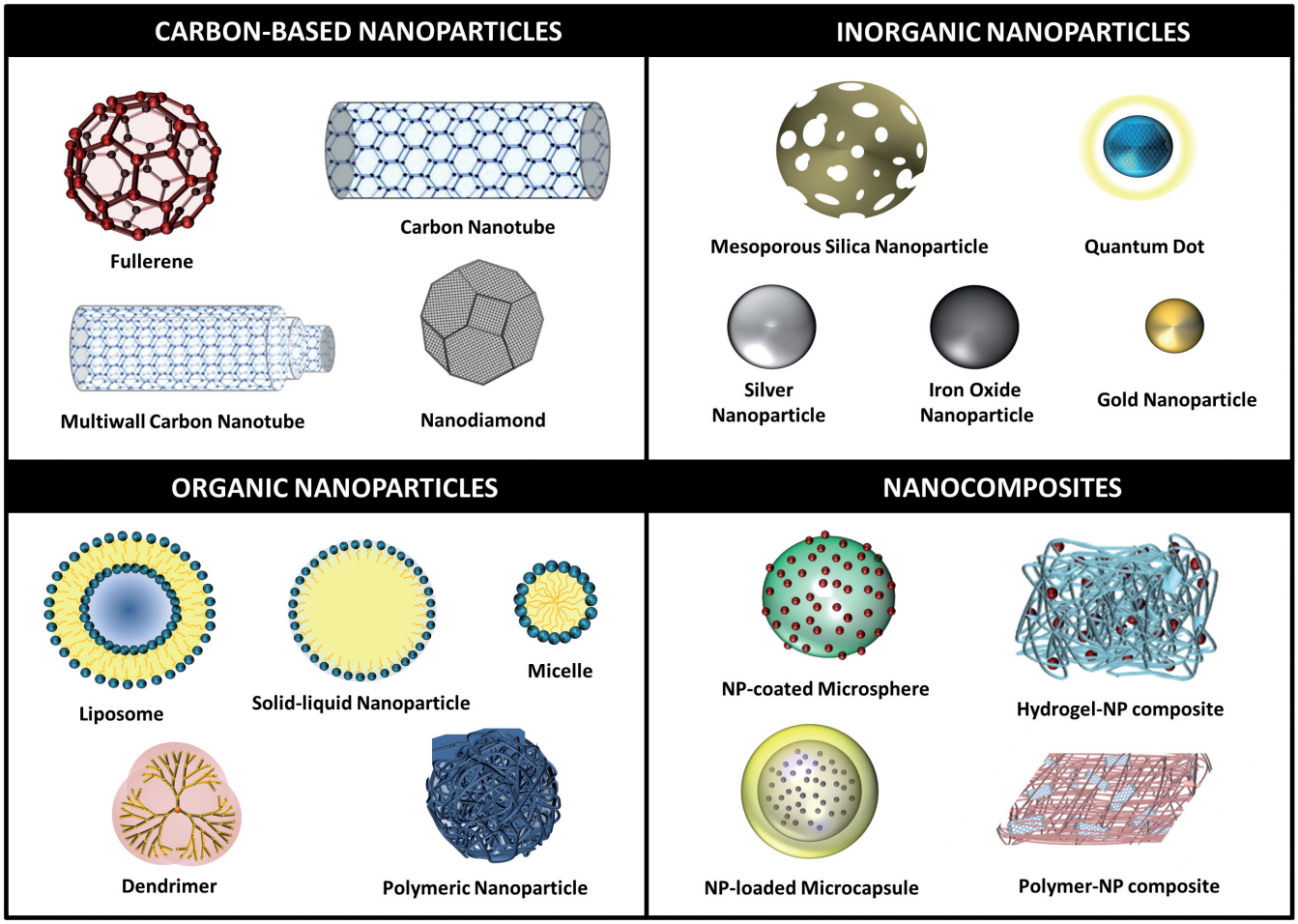

Fig. 1. Main categories of nanoparticles based on their constituents. NPs can be divided into: carbon-based, inorganic, organic and composite. Carbon-based NPs include fullerenes, carbon nanotubes and nanodiamonds. Inorganic NPs include quantum dots, mesoporous silica and metal-based NPs. Organic NPs include liposomes, micelles, dendrimers, and polymeric NPs. Nanocomposites derive from combination of carbonic, metallic or organic NPs with any form of ceramic, metallic, or polymer bulk materials.

ceramic NPs are endowed with low biodegradability, high mechanical strength and good body response (Thomas et al., 2015; Singh et al., 2016). Inorganic NPs are promising to advance and replace conventional surgical materials in hard and soft tissue repair (Urie et al., 2018). Metallic, mineral and ceramic NPs have all shown capability to enhance scaffold functionality in different ways (Lewandowska-Łańcucka et al., 2015; Urie et al., 2018; Huang and Chu, 2019), via optimizing either its structural features (such as rigidity, topography, …) or other physical properties (electrical conductivity, intrinsic magnetism). Moreover, they have been applied in cellular therapy to control the environment, engraftment, and biology of stem cells. For example, mesoporous silica NPs have been the object of several studies focused on osteogenic differentiation of stem cells and bone tissue formation (Rosenholm et al., 2016). The electrical signal transfer among neural cells can be enhanced by prompting the electrical conductivity of scaffolds through metallic NPs, such as Zn or Au-based ones (Aydemir Sezer et al., 2017; Saderi et al., 2018). The AuNP-mediated electrical stimulation can exert a crucial role also in the maturation of cardiac progenitor cells (Ravichandran et al., 2014; Solazzo et al., 2019).

Organic NPs

Excluding those belonging to the previous categories, all NPs predominantly composed of organic matter are organic NPs. They can be composed of macromolecules such as polymers or dendrimers, or be the consequence of self-organization (micelles, inverted micelles, liposomes, polymersomes, dendrimersomes and capsules).

Bioactive molecules (e.g. drugs) and labels (e.g. fluorescent proteins) can be loaded onto these NPs by chemical conjugation on the surface, incorporation into the NP matrix, or physically encapsulated into the inner cavities. The predisposition to chemical or physical manipulation makes these systems a favoured tool for targeted delivery of genes, drugs, sensing and targeting agents (Parat et al., 2014), and other biological material, in order to detect, prevent and treat complex diseases (Park and Na, 2015; Palazzolo et al., 2018). Advanced NPs have been developed to enable the spatially and temporally controlled release of therapeutics in response to specific stimuli at disease sites. For instance, by virtue of their high temporal and thermal stability, loading capacity, low production costs, ease of preparation, and large-scale industrial production from natural sources, the lipid-based nanoparticle (LBNP) systems have improved the antitumor activity of several chemotherapeutic agents *in vitro* and *in vivo*, with promising results also in some clinical trials (García-Pinel et al., 2019). Organic dyes can also be transformed into NPs using various strategies, obtaining colloidal effectors of clinical phototheranostics (Braeken et al., 2017; Cai et al., 2018; Zhu et al., 2019). Polymers and conjugated polymers display optical and semi-conducting attitude, becoming useful in photodynamic and photothermal therapy, as well as in molecular bioimaging (Li et al., 2018a). However, since the basic units for the generation of these supramolecular entities are organic, polymeric or dendrimeric molecules with various structures but often large in size, the size control of the final object can be difficult especially in the simpler systems (Romero and Moya, 2012). Moreover, many of these soft particles are of unstable and fluid nature, subjected to phenomena of dynamic interaction typical of colloidal suspensions, such as molecular re-arrangements of the architecture, fusion, and fragmentation. Given that a number of physical characteristics (including size, surface characteristics, arrangement of layers, and homogeneity) determine the *in vivo* behaviour, the morphology control is of key importance for an effective clinical translation (Robson et al., 2018).

Composite nanomaterials

Nanomaterials with heterogeneous composition, consisting of different phases, are referred to as composite nanomaterials (De Cicco et al., 2017). Any combination of carbon, metallic or organic NPs with any form of ceramic, metallic, or polymer bulk materials represents a multiphasic system with at least one phase on the nanoscale dimension. This composite is obtained by combining NPs with other NPs, larger bulk-type materials or differently structured architectures, like organic frameworks. For instance, carbon-based NPs (CNTs, CNFs, and GNPs) can be included into polymeric matrices to improve various properties (e.g., mechanical, electrical, and thermal) of their host polymers (Choi et al., 2005; Alishahi et al., 2013; Gao et al., 2014). Similarly, also the incorporation of metallic NPs improves electrical, optical, magnetic, and mechanical properties of polymers with strong impact on their application into biomedical sciences (Chou et al., 2006; Kucheryavy et al., 2013; Sardana et al., 2014). Mineral NPs (like nanoclays and nanosilicas) are reported to enhance the mechanical properties, fire resistance, liquid infusion, strength, flexibility, and durability of polymers (Javni et al., 2002; Ji and Li, 2008). Hydrogels, namely physically or covalently crosslinked networks of hydrated polymers swollen with water, can be loaded with a wide array of nanostructures, being structurally reinforced and gaining responsiveness to external stimuli (Biondi et al., 2015; Dannert et al., 2019). Composites between NPs and nanofibers originate scaffolds with a number of applications in tissue engineering (TE) (Mortimer and Wright, 2017). Owing to its osteoconductive properties and biocompatibility, nano-hydroxyapatite (nHA) as bioceramic material has been associated to polylactic acid, polycaprolactone, poly(lactic-co-glycolic) acid, polyamide, polyvinyl alcohol, polyurethane, polyhydroxybutyrate, chitin, chitosan, collagen, gelatin, and fibrin to create scaffolds used as bone graft

substitute (Venkatesan and Kim, 2014). Photocatalytic and fluorigenic nanocomposites have also been reported (Matejka and Tokarský, 2014; He et al., 2015).

Despite the notable progress achieved in optimizing NP application in a wide variety of scientific research fields over the last two decades, their use in TE is still highly limited. In the following paragraphs, we review the use of various types of NPs in TE and the advantages and limitations associated to the specific intrinsic properties of each material.

Nanoparticles in tissue engineering

The use of NPs in TE and regenerative medicine raised great expectations (Rudramurthy and Swamy, 2018). In regenerative medicine, a series of synchronized events occurring among the cellular components of the implanted material, the circulatory system and the host immune system rule the *in vivo* maturation of engineered tissues. Indeed, the approaches of nanobiomimetics offer an attractive route towards the fine tuning of physicochemistry of TE materials (Singh et al., 2014). The conventional functions of NPs (such as viral transduction, gene delivery, DNA transfection, biosensing and molecular detection) were adapted and dedicated to TE purposes, while other functions were developed specifically for TE. The novel functions of NPs aim at ameliorating the manufacturing and the biological, electrical and mechanical performances of scaffolding materials, and at modulating the responses by host cells involved in regeneration (Fathi-Achachelouei et al., 2019). In fact, to obtain a precise control over tissue maturation which affects the integration and long-term safety of implanted materials, several nanoscale objects are employed to render them antimicrobial or more biologically active, to modulate their structural properties, to allow their monitoring over time by concentrating contrast agents, to trigger sequential release of several growth factors, to induce surrounding tissue reactions, or to improve bioprinting approaches. Ceramic, metallic, polymeric and magnetic/paramagnetic NPs are protagonists in TE field, constituting a crucial toolbox for directing the engineering of artificial organs and organoids. Their action could be exploited to have direct effects on cell behaviour (Kerativitayanan et al., 2015; Dayem et al., 2016; Ishihara et al., 2018; Kerans et al., 2018), or on the structural and functional properties of supporting materials (Rosenholm et al., 2016; Mortimer and Wright, 2017; Gorain et al., 2017; Saleh et al., 2018). Specifically, because of their structural similarity to native tissues, polymeric hydrogels have been extensively modified for TE purposes by incorporation of noble metal particles, particularly Au, Ag, or of Fe NPs (Song et al., 2015; Min et al., 2018; Tan et al., 2019). This association finds application in the engineering of soft tissue as well as in hard ones such as bone tissue, and are achieved by: (i) crosslinking of the hydrogel in a NPs/polymer mixture, (ii) *in situ* synthesis of NPs within the hydrogel matrix, (iii) *in situ* synthesis of NPs during hydrogel formation, and (iv) crosslinking of hydrogels by NPs (Tan et al., 2019). Also carbon NPs and conductive polymers can be integrated into hydrogels to create conductive hybrid gels useful in cardiac, nerve, and bone TE (Min et al., 2018). Every tissue presents different challenges, requiring therefore the identification of specific nanotechnological therapeutic strategies designed to mirror the biological needs of a specific organ (Singh et al., 2014). Below, we discuss the recent progress of nanobiotechnology in TE of the skeletal apparatus, focussing on its three main functional tissue compartments, namely bone, cartilage and vasculature (Fig. 2).

Bone tissue

Bone is a tissue with excellent self-regenerative capability which, however, can be lost upon serious damage/disease (e.g., trauma, infection, arthritis) (Li and Liu, 2017). Scaffolds loaded with cells and containing growth factors are cultured *in vitro* to allow for cell proliferation before being implanted in humans for guidance of bone regeneration. This process highly depends on the assembly of inorganic nanocrystals with organic nanofibers, a process known as "biomineralization". By being comparable in size to structures appearing in natural bone and able to closely mimic the architectures and mechanisms observed in naturally occurring systems, nanomaterials with intrinsic potential of biomineralization both *in vitro* and *in vivo* have been developed (Saiz et al., 2013). Large superficial area, surface roughness and affinity towards biological factors have made hydroxyapatite (HA) NPs a primary material to design composite scaffolds for bone TE (BTE), demonstrating ability to improve growth, cell adhesion, interaction and differentiation of osteoblasts (Venkatesan and Kim, 2014). However, a substantial advancement of scaffold properties has been achieved by introducing alternative nanomaterials prepared from polysaccharides, carbon-derived molecules, TiO_2, synthetic polymers, and others (Saiz et al., 2013; Dhivya et al., 2015; Li and Liu, 2017). Several of these innovations find application in craniofacial and dental reconstruction (Li et al., 2017). In general, in BTE, the NPs are mainly dedicated to: (i) drug and gene delivery carriers, (ii) cell labelling and (iii) scaffold modification.

NPs for gene and drug delivery

In the complex cascade of biological events driving bone regeneration, different growth factors mediate biological activities via intracellular and/or extracellular molecular signalling pathways. Among these regulators, bone morphogenetic protein-2 (BMP-2) and BMP-7 are key growth factors able to guide the directional osteogenesis by stem cells (Rosen, 2009). Moreover, given that after transplantation, the viability of cells and

new formed tissue strongly relies on the timely formation of a vascular network supplying sufficient nutrients and oxygen, vascular endothelial growth factor (VEGF) is also important due to its strong angiogenic properties (Kanczler and Oreffo, 2008). In order to functionally mimic the natural extracellular matrix (ECM) and sustain the release of crucial molecules with a precise temporal and spatial pattern, multiple signalling agents (such as growth factors and chemokines) can be associated to particulate materials in order to drive the process of tissue repair (Gu et al., 2013). BMP-2, VEGF, Insulin growth factor 1 (IGF-1), dexamethasone (DMX), deferoxamine, alendronate, human parathyroid hormone (PTH (1-34)), and others are commonly loaded into NPs intended for bone drug delivery. Conventional gene therapy effectors (namely, plasmids, miRNA, shRNA, siRNA, etc.) can also be loaded and conveyed by nanocarriers, and are often involved in balancing the interplay between bone-resorption and formation (Wang et al., 1997). For instance, next to widely used polymers (like polyacrylates, polyglycolides, and polycaprolactone), the copolymer poly(lactic-co-glycolic acid, PLGA) generates suitable NPs for bone regeneration by virtue of its carboxylate ending groups that confer hydrophilicity to the surface. Also its tuneable properties allow for easy functionalization and bone-specific drug delivery (Choi and Kim, 2007; Jayaraman et al., 2015; Gentile et al., 2015). Besides several examples of PLGA-derived systems (Choi et al., 2014; Ortega-Oller et al., 2015), also other NPs have been reported as delivery carriers for bone-stimulating factors (both in form of drug and genetic material), such as chitosan, collagen and silicate NPs (Hong et al., 2017; Bastami et al., 2017; Yao et al., 2018). For example, upon biodegradation, chitosan NPs supplied stable release of BMP-2 from titanium implant substrates (Poth et al., 2015), and miR-199a-5p agomir to boost the osteogenesis of human mesenchymal stem cells (MSCs) via the HIF1α pathway (Chen et al., 2015). BSA NPs were embedded into electrospun nanofibers allowing for the controlled dual delivery of BMP-2 and DMX in critical-sized rat calvarial defect (Li et al., 2015). BMP-4 protein fused to a nanosized carrier derived from fibronectin, the collagen-binding domain (CBD), was better retained in the bone, causing augmented osteogenic responses in the absence of a scaffold *in vivo* (Shiozaki et al., 2013). Polymersomes for delivery of Wnt-activating small molecules promoted the osteogenic differentiation in human primary bone marrow stromal cells (BMSCs) (Scarpa et al., 2018). Polyurethane (PU) nanomicelles modified by the acidic peptide Asp8 specifically delivered the therapeutic

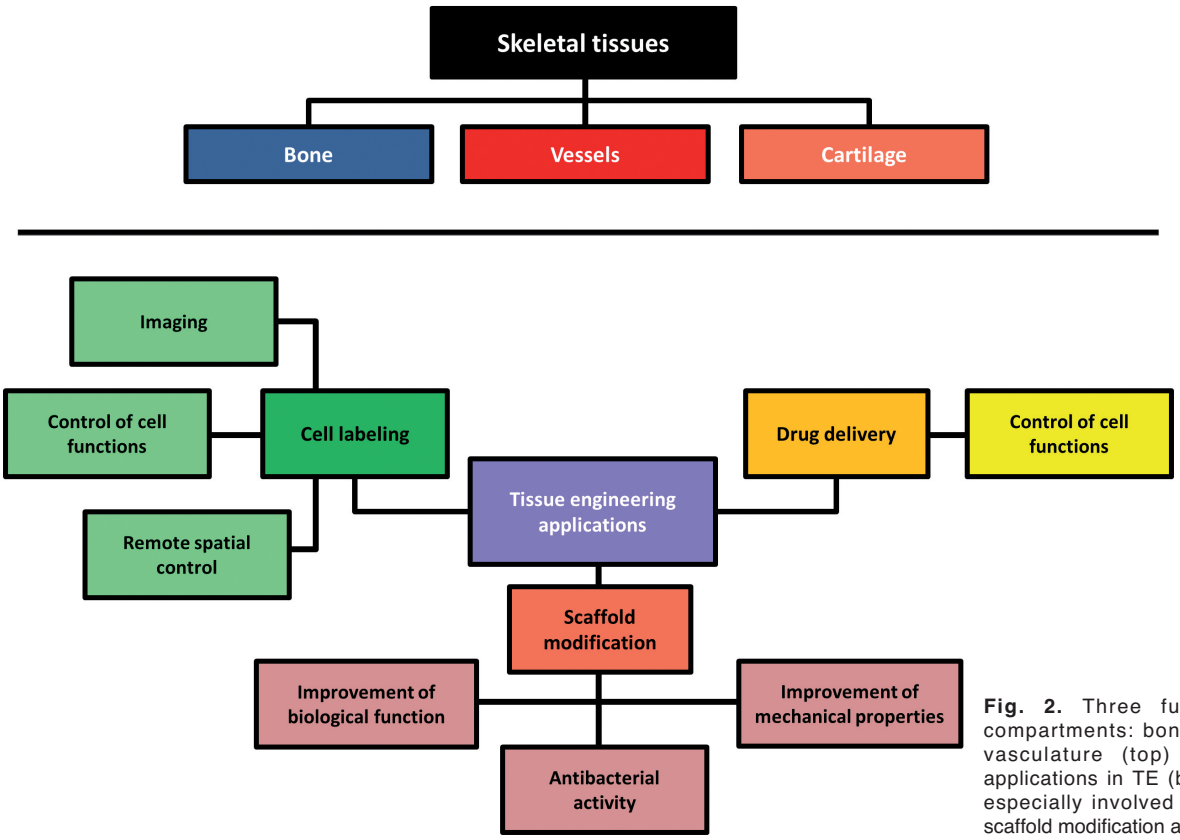

Fig. 2. Three functional tissue compartments: bone, cartilage and vasculature (top) and main NP applications in TE (bottom). NPs are especially involved in cell labelling, scaffold modification and drug delivery.

miRNA anti-miR214 to osteoclasts, improving bone microarchitecture and bone mass in osteoporosis murine models (Cai et al., 2017). The same group reported a similar system for gene therapy targeting the osteoblastic activity (Sun et al., 2016). NDs functionalized with DMX modulated gene expression, cell area, and focal adhesion in human adipose stem cells (hASCs) into hydrogel scaffolds (Pacelli et al., 2017). Also, alendronate-modified liposomal NPs (Aln-Lipo) carrying SDF-1 gene presented an increased mineral binding affinity, facilitating the gene delivery to osteoblastic cells. The up-regulated SDF-1 expression in osteoblasts triggered MSCs migration, resulting in their accumulation in osseous tissue in mice (Chen et al., 2018). Becoming popular tools in BTE (Kong et al., 2018), the bioactive glass nanoparticles (BGNs) in the form of mesoporous nanospheres with a composition of 15% Ca-added silica (proven to be bone-bioactive) were loaded with a BMP2 plasmid, showing better internalization and osteogenic development in MSCs (Kim et al., 2016a). BGNs complexed with siRNA inhibiting the expression of the receptor activator of nuclear factor kappa B (RANK) also suppressed osteoclastogenesis (Kim et al., 2016b). With respect to viruses and other vectors, NPs are characterized by potentially higher safety, degradability, and wider versatility in surface modifications. Furthermore, they also offer prolonged therapeutic effects compared to smaller carriers (such as pure proteins) or drug administration in form of small molecules. They moreover can be easily applied topically into cements, implants, or even directly injected into bones, minimizing systemic toxicity (Tautzenberger et al. 2012).

NPs for cell labelling

Cell labelling with NPs offers great potential for the detection of transplanted cells, via various imaging techniques, during regenerative therapy (Edmundson et al., 2013). Depending on the therapeutic approach, the binding of cells with NPs can occur either directly *in vivo* after systemic administration of the nanoformulation, or during a preliminary phase of *ex vivo* labelling, before the cells are applied to the host organism. In particular, MSCs display exceptional bone regenerative potency, promoting tissue regeneration and positively impacting on the fracture healing (Edmundson et al., 2013). Upon labelling, MSCs can be traced and monitored *in situ* over time. Four main categories of labelling agents have been developed for this purpose. They include: (i) superparamagnetic iron oxide nanoparticles (SPIONs), (ii) Au NPs, (iii) mesoporous silica NPs, and (iv) QDs. Even though a variety of SPIONs are well-established contrast agents for Magnetic Resonance Imaging (MRI) and have already been clinically approved for visualization of many tissues (Jendelová et al., 2003; Bashir et al., 2015), bone represents a challenging target organ because its high mineral content complicates the detection of labelled cells. However, consisting of iron oxide cores, SPIONs can be targeted to bone through application of magnetic field gradients to increase the local density of NPs (Iyer et al., 2017). Moreover, to allow NPs to reach deeper areas inside bone, magnetic fields can be used to better direct NPs (Huang et al. 2010). Such technology may become relevant in BTE, where site-specific regeneration is required, by driving cell homing to challenging sites such as bone and facilitating the engraftment.

Whereas several types of NPs are suitable for *in vivo* cell tracking, there is a growing interest to define whether they may affect cell responses, especially by interfering with biological functions, such as the differentiation capacity of progenitor cells used for regenerative purposes. The results regarding the modulatory effect of the NP labelling on the osteogenic potential are still ambiguous. Shao et al. (2017) reported that carbon dots are capable of both fluorescently tracking MSCs and enhancing their differentiation potential. Nanobioactive glass, silver NPs and graphene oxide QDs improved osteogenic development (Liu et al., 2014; Wang et al., 2014; Yang et al., 2017). In contrast to Au NPs (Ricles et al. 2011) and mesoporous silica NPs (Chung et al., 2007) which seem not to affect osteogenesis by MSCs, CdSe/ZnS QDs tend to inhibit it (Hsieh et al., 2006). By increasing the size and concentration, also calcium phosphate NPs impaired osteogenic cell differentiation and matrix mineralization, especially when amorphously structured as compared to a crystalline form (Hu et al., 2007; Liu et al., 2009). Some *in vitro* studies have shown no effect of SPIONs on osteoprogenitors' differentiation (Farrell et al., 2008). Such *in vitro* results do not necessarily reflect the possible impact in *in vivo* models. Some groups observed a higher differentiation capacity by labelled human MSCs in an immunocompromised mouse model (Balakumaran et al., 2010) and a positive influence of SPIONs on bone regeneration in rats (Hu et al., 2018), whereas reduced bone forming capacity has been claimed elsewhere (Chen et al., 2010). In general, SPIONs are assumed to alter osteogenic MSC fate, likely through an increased iron intracellular content (Farrell et al., 2008). Beyond differentiation, other MSC functions can be modulated: for instance, proliferation is stimulated by SPIONs via reduction of intracellular hydrogen peroxide (Huang et al., 2009), while commercially pure titanium NPs decreased not only differentiation but also adhesion (Okafor et al., 2006). Finally, iron oxide-based NPs offer the unique chance to magnetomechanically actuate labelled cells (Lamanna et al., 2013), to regulate their spatial distribution or to activate mechanosensitive signalling pathways (Li et al., 2018b; Liu et al., 2018). On one side, the exposure of magnetically labelled cells to external magnetic gradients allows for remote control of their spatial distribution with important implications for 3D bioprinting of engineered materials (Buyukhatipoglu et

al., 2010). On the other side, the activation of specific mechanotransduction membrane receptors is also possible when SPIONs are conjugated with targeting antibodies to apply mechanical force directly to stretch-activated ion channels on the cell surface, leading to stimulation of intracellular molecular pathways with impact on osteogenic development (Henstock et al., 2018).

Successful bone tissue reconstruction is dependent on a close interaction among all bone cellular components (bone-forming osteoblasts and bone-resorbing osteoclasts). Therefore, it is important to study the possible particle uptake by those cells and its related effects. Studies about the influence on differentiation and mineralization of osteoblasts are divergent depending on NP characteristics (material, shape, charge, size). Calcium phosphate nanoshells increased several markers of osteoblast differentiation, such as alkaline phosphatase (ALP) activity, calcium deposition and collagen type I synthesis (Schmidt et al., 2008). While similar effects were obtained with HA-coated IONPs (Tran and Webster, 2011) and Sr-doped HA nanocrystals (Capuccini et al., 2009), Ag and Ti NPs were found to downregulate the expression of ALP (Lohmann et al., 2000; Albers et al., 2013). NPs can also influence osteoclast-regulating genes. Polyethylene (PE) NPs altered the RANKL/osteoprotegerin ratio (Atkins et al., 2009) while Ti NPs stimulated gene expression of granulocyte colony-stimulating factor (g-CSF) (Zhang et al., 2011). Deriving from the same precursors as other phagocytizing cells, such as macrophages, the osteoclasts naturally internalize NPs, which affects the formation of osteoclasts and their bone resorption activity (Wang et al., 1997, 2019). Rod-like HA NPs were shown to differentially affect osteoclastic differentiation and function of peripheral blood mononuclear cells (PBMC) depending on the dose, the administration time and the differentiation stage (Costa-Rodrigues et al., 2014), whereas their exposure to subtoxic concentrations of Ag NPs did not induce changes, neither in differentiation nor in podosomal structures (Pauksch et al., 2014). BGNs can control gene expression in murine macrophages and human osteoclasts in a concentration-dependent manner, and can stimulate osteoclastogenesis (Detsch et al., 2016). Synthetic mineral NPs, such as whitlockite NPs, impede osteoclastic activity and continuous supply of PO_4^{3-} and Mg^{2+} drives *in situ* remodeling and rapid bone regeneration (Kim et al., 2017a). Some metallic NPs limit the *in vitro* formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclastic cells (Sul et al., 2010) and can suppress osteoclast maturation in a dose-dependent manner, leading to higher bone density in osteoporotic murine models (Lee et al., 2016). Other metallic NPs can in contrast boost the resorption activity of both undifferentiated monocytes and osteoclasts (Cadosch et al., 2009). Similarly, some silica NP formulations inhibit osteoclast maturation (Beck et al., 2012), whereas others can either promote it (Nabeshi et al., 2011) or have no effect on the formation of multinucleated cells (Tautzenberger et al., 2011). In summary, NPs have contrasting effects on bone forming and resorbing cells, which should be taken into account when labelling bone tissue.

Scaffold modification

Scaffolds are biocompatible and biodegradable supports that can be ultimately replaced by functional tissue. Frequently, NPs are combined with scaffolds, such as protein hydrogels or degradable polymeric matrices, in order to prompt regeneration upon bone grafting (Singh et al., 2014; Song et al., 2015; Rashkow et al., 2018). It can improve the mechanical properties and the biological functionality of the implanted constructs, with effects on cell survival, proliferation, adhesion, differentiation, and integration into the surrounding environment (Yunus Basha et al., 2015; Corona-Gomez et al., 2016; Gera et al., 2017). The surface topography of the scaffold is modified with NPs to enhance the physicochemical interaction between the native *in vivo* environment and the implanted material (Bacakova et al., 2011). For instance, enrichment with calcium phosphate NPs improves cytocompatibility of biodegradable scaffolds for dynamic seeding of MSCs (Sauerova et al., 2019). By mimicking the native mineral crystal reticulate of bone, various biomimetic apatite particles can stimulate the osteogenic capacity of progenitor cells, also fostering survival and extension of cellular projections along textured surfaces (Chou et al., 2005). The combination of HA NPs with polyamine (PA) scaffolds was also shown to upregulate the expression of osteogenic proteins (i.e. BMP-2, osteoprotegerin, osteopontin, collagen type I, and osteocalcin) in rabbit bone marrow-derived MSCs, facilitating bone regeneration in critical-sized mandible defects (Guo et al., 2012). In a similar fashion, HA coating onto PLGA 3D scaffolds stimulated preosteoclast differentiation *in vitro* (Chou et al., 2005) and accelerated bone deposition by local progenitors in rat calvarial defects (Kim et al., 2007). The hybridization of HA on other substrates, such as alginate, confers unique nanofibrous topography advantageous for bone regenerative medicine applications (Chae et al., 2013). As for PA matrix-HA nanocomposites (Guo et al., 2012), also those gathering carbon nanotubes demonstrated to be ideal substrates for MSC adhesion and to stimulate osteoprogenitor differentiation (Ciapetti et al., 2012). Graphene oxide nanoplatelets (GONPs), molybdenum disulfide nanoplatelets (MSNPs), or tungsten disulfide nanotubes (WSNTs) dispersed in PLGA showed good bioactivity *in vitro*, holding promise for *in vivo* tissue repair at the bone-implant interface. In 3D scaffolds based on a nanofibrous structure incorporating BGNs, the release of Si and Ca ions at biologically effective levels and nanofibrous topology synergistically promoted osteogenesis and angiogenesis (Kim et al., 2017b). Upon

association with metallic NPs, scaffolds also gain better bone forming capacity thanks to advanced mechanical strength and cellular attachment (Sahmani et al., 2019). Zinc oxide NPs incorporation enhances porosity, tensile modulus and cytocompatibility of chitosan-collagen 3D porous scaffolds (Ullah et al., 2017). Assemblies of hematite NPs were used to coat the surface of polymer electrospun scaffolds, creating an osteoinductive interface acting on rat adipose-derived stem cells (ADSCs) (Ma et al., 2019). Moreover, the boosted alkaline phosphatase activity, collagen synthesis, and calcium deposition in osteoblasts exposed to iron, titanium, and alumoxane can determine higher tensile strength of the overall construct (Horch et al., 2004; Goto et al., 2005; Tran and Webster, 2011). Keeping in mind that, in addition to chemical substances, also mechanical forces play a major role in determining cell functions, such modulation of the cell behaviour possibly finds its main underlying mechanisms in the osteoinductive action triggered by the augmentation of nanoscale surface roughness. The relevance of surface topographic cues in regulating the response of native cells to scaffold implantation has also been well documented for diverse NP-mediated modifications (Bacakova et al., 2011). Similar to the shear stress exerted by bioreactors, also the stiffness of patterned substrates offers the chance to modulate mechanotransduction-related intracellular pathways. A step further in these approaches is represented by the use of magnetic NPs which can be spatially and temporally guided through a magnetic field (Santos et al., 2015; Xia et al., 2018). An example is the incorporation of IONPs which allows for remote control of the overall scaffold architecture (Li et al., 2018b), enabling the trigger of drug and cell delivery from biphasic ferromagnetic hydrogels under application of external magnetic forces (Cezar et al., 2014). The combination of magnetic NPs with thermo-responsive polymers also generates smart nanoengineered hydrogels for on demand release of biomolecules. Under exposure to alternated magnetic fields (AMF), the magnetic nanostructure of these gels produces radiofrequencies resulting in thermally-induced conformational changes of the polymers and eventually leading to the release of the entrapped drug. These systems are becoming available in a range of biomedical applications, including therapeutic delivery, bioimaging, and regenerative engineering (Jalili et al. 2016). In 2013, Gloria et al. developed fully biodegradable and magnetic BTE nanocomposite substrates by embedding iron-doped hydroxyapatite (FeHA) NPs in a poly(ε-caprolactone) (PCL) matrix, displaying the ability to support osteogenic differentiation. Increasing evidence further demonstrates the effective osteogenic induction by magnetic composite bone scaffolds in both *in vitro* and *in vivo* systems (Li et al., 2018b).

In addition to scaffold modification, the advances achieved in the process of scaffold fabrication itself may offer new opportunities for TE. Electrospinning allows for nanofiber alignment, acting as guidance for controlling the cell behaviour, conduction of electrical stimuli and regulation of muscle-skeletal biomass homeostasis (Chen et al., 2013). Moreover, electrospun nanofibers biodegradable synthetic polymers trigger little immunogenic response, possibly avoiding or reducing the risk of premature degradation of scaffolds (Lee and Shin, 2007). The dispersion of HA or biphasic calcium phosphate (BCP) NPs was shown to be useful in strengthening the tensile force of electrospun materials, in the effort to expand the applicability of chitosan, silk fibroin, polyvinyl alcohol and other materials with low mechanical strength, to BTE (Linh et al., 2013; Kim et al., 2014). Since cell penetration and tissue integration can be prevented by the relatively tight lattice network of electrospun nanoscaffolds, alternative manufacturing approaches are emerging. For instance, nanofiber airbrushing is a technique capable of synthesizing open structure nanofiber scaffolds at high rates, where compressed gas is used to blow polymer solution through a small nozzle which shears it into fibers (Hoffman et al., 2015).

Cartilage tissue

Hyaline cartilage is a connective tissue based on a single cell type (chondrocyte), embedded within an ECM mostly consisting of glycosaminoglycans (GAGs) and type II collagen (Camarero-Espinosa et al., 2016). Acting as a cushion reducing the friction and load-bearing effects derived from skeletal movements, it is composed of three distinct layers, differing based on ECM content, structure, and cell behaviour. In general, cartilage is characterized by minimal cellularity, vascularization, and proliferative capacity of the residing chondrocytes and has very limited self-repair capacities. Recently, more and more inputs from nanomaterial science were introduced to reinforce TE approaches (Chen and Kawazoe, 2018; Manoukian et al., 2018). In particular, nanomaterials enabled for the simulation of nanoscale features found in the native ECM (Manoukian et al., 2018). As for BTE, in cartilage TE (CTE), nanomaterials assist drug and gene delivery, cell labelling, as well as scaffold engineering. Special emphasis was given to the development of hydrogel systems, due to the injectable formulation and to the versatility and the ease of NP-coupling (Asadi et al., 2018; Jeznach et al., 2018). The exposure of chitosan/hyaluronic acid NPs to pellet co-cultures of articular chondrocytes (ACs) and infrapatellar fat pad (IPFP)-derived mesenchymal stem cells (MSCs) improved the production of type II collagen and aggrecan (Huang et al., 2017). This mixture moreover prevented the hypertrophic differentiation of MSCs and the inflammatory status of ACs, which occurs in knee osteoarthritis (OA). When seeded with chondrocytes and transplanted into articular cartilage defects, electrosprayed NPs composed of cartilage specific

proteins (namely hyaluronic acid and type II collagen) supported cell survival and functionality (Yang et al., 2018). In 2014, Zhao et al. addressed the same issue with a NP-mediated gene delivery approach. Briefly, through a complex coacervation-based encapsulation process, chitosan-plasmid DNA (pDNA) NPs encoding shRNA targeting MMP-3 and -13 were obtained and used to transfect expanded chondrocytes and to silence dedifferentiation-related genes. Indeed, various NPs loaded with bioactive agents have been employed in order to prompt neo-connective tissue formation (Monteiro et al. 2015). ADSCs can be transfected with BMP-4 plasmid through PLGA NP-mediated delivery, going towards chondrogenesis *in vitro* and in rabbit articular defect model (Shi et al., 2013). Local delivery of TGFβ-1, TGFβ-3, IGF-I and other growth factors was achieved by a variety of nanocomposite structures including PLGA microspheres coated with poly-lysine NPs, fibrin hydrogel mixed with heparin NPs, PLC scaffolds produced by a gel-pressing method with PLGA/Pluronic heparin NPs and nanofibrous scaffolds prepared by coaxial electrospinning with embedded liposomes (Park et al., 2008, 2009; Jung et al., 2009; Mickova et al., 2012). NPs and nanocomposites are also substantially involved in applications related to osteochondral regeneration and repair (Oliveira et al., 2018), where several scaffolds integrating nanomaterials have been already proposed (Xiao et al., 2019). Finally, labelling with magnetic NPs allows for spatial control and imaging of cells involved in injured cartilage treatment. For instance, magnetic-assisted delivery of MSCs led to cartilage repair in minipig models, where full-thickness defects were operated in the centre of patella and the accumulation of magnetic cells was driven by a localized 1.5 T magnetic field (Kamei et al., 2013). Concerning more specifically the imaging studies, it was established that optimal low dosages of SPIONs enabled chondrocyte MRI-tracking in large animal models while maintaining essential cell properties (Chen et al., 2012). Moreover, labelling and imaging of hMSCs transplanted for cartilage regeneration in arthritic joint has also been performed. It was found that labelled hMSCs are phagocytosed by macrophages when undergoing apoptosis, determining a faster disappearance of the MRI signal as compared to viable labelled cells (Nejadnik et al., 2016). Given that in this case poor cartilage repair outcomes are observed, NPs acquire also a predictive function on the incomplete defect repair weeks later. Chondrocytes labelled with magnetic NPs seeded on a biphasic, type II collagen-chitosan/PLGA matrix enabled image-monitoring of cartilage regeneration and osteochondral defect repair (Su et al., 2017). Finally, NPs being visible in bimodal MRI/IVIS imaging were also developed. Composed of $Gd_2(CO_3)_3$, these novel contrast agents proved their ability to target cartilage effectively, and to specifically convey hesperetin to protect chondrocytes from apoptosis and inflammation via TLR-2/NF-κB/Akt signaling (Ouyang et al., 2019).

Vessels

Even though cartilage tissue displays poor vascularization, bone tissue is instead densely perfused. Blood vessels play crucial roles in controlling multiple aspects of bone formation and healing, and supply niches for hematopoietic stem cells resident within the bone marrow (Sivaraj et al., 2016). In bone, vessels originate through a process of angiogenesis following bone-specific mechanisms. The result is a network of arteries and arterioles with distinct molecular hallmarks and distribution, which guarantees a constant source of oxygen, growth factors, nutrients, hormones, and neurotransmitters to the skeletal system. Most cells that are farther than 200 μm from capillaries cannot survive due to lack of nutrients and oxygen. Therefore, the successful outcome of bone grafting or bone tissue engineering-based strategies largely depends on a proper blood supply and timely regeneration of vasculature (Filipowska et al., 2017). Novel multifunctional nanocomposite scaffolds for advanced bone TE combine double activities by simultaneously tuning the osteogenic and angiogenic capacity of seeded cells (Cattalini et al., 2016; Yegappan et al., 2019). Angiogenesis is often triggered by conveying chemical cues to endothelial progenitors with certain nanoparticulate objects (Kim et al., 2017c; Strobel et al., 2018). Common chemical stimulators include angiogenic factors (such as VEGF) and drugs (such as dimethyloxallylglycine), as well as metals (e.g. copper ions). The most popular strategy relies on conveying VEGF or its gene into cells. For example, smart and temperature-sensitive poly(N isopropylacrylamide) (PNIPAM) NPs integrated into collagen hydrogel and loaded with VEGF affected both angiogenic and osteogenic differentiation of human bone marrow derived mesenchymal stem cells (hBMSCs), as assessed at the gene expression level and by cell morphological observation (Adibfar et al., 2018). Recently, superparamagnetic microspheres have been loaded with plasmids and their movements within the scaffold were induced by an oscillating and a static magnetic field. It caused the detachment of the plasmids and the transfection of the surrounding cells, resulting in expression of VEGF inside a large bone defect repair model (Luo et al., 2018). Besides producing an osteoinductive environment for rat ADSCs, the incorporation of whitlockite NPs and dimethyloxallyl-glycine into carrageenan nanocomposite hydrogel also elicited enhanced cell migration and capillary tube-like structure formation by human umbilical vein endothelial cells (HUVECs) (Yegappan et al., 2019). Hybrid particles of reduced graphene oxide decorated with Cu NPs embedded in PCL matrix released copper and induced pro-angiogenic, osteogenic and bactericidal properties to endothelial cells, pre-osteoblasts and *E. Coli*, respectively (Jaidev et al., 2017). Interestingly, some nanomaterials have advantageously modified the properties of additive substances used in regeneration

and scaffold engineering. For instance, nano-fibrin can transform the morphology of $CaSO_4$ crystals, which are popular biodegradable bone-forming agents, commonly added to regenerative gels, from needle to hexagonal geometry. In injectable composite chitosan formulations, this morphological switch of the crystals improves the rheology and angiogenic potential of the gel. Nano-fibrin also masked the retarding effect of the crystals towards *in vitro* early cell attachment and endothelial organization (Arun Kumar et al., 2016).

Clinical perspectives

Even though the formulations of NPs under clinical examination or approved for human use are several, these studies are for the most pursued in the scope of diagnosis and treatment of cancer or other critical diseases (Kalita et al., 2014; Nag and Delehanty, 2019), especially in relation to NPs' ability for active targeted drug delivery and image-guided tracing of cells (Liang et al., 2018; Yahyapour et al., 2018; Savitsky and Yu, 2019; Zhang et al., 2019). Wound healing is also an active clinical arena for various kinds of NPs (Oyarzun-Ampuero et al., 2015; Lee et al., 2017). However, the clinical validation of NPs in TE has just begun. Both effectiveness and toxicity in humans are key for a successful translation of NPs in the future. Only few human studies have tested the safety of nanostructured biomimetic scaffolds and the feasibility of the surgical procedure required for their implantation (Kon et al., 2010, 2011). One of the main areas of clinical development is the repair of osteochondral and bone defects, where tested materials are mostly nanocomposites enriched with HA NPs (Kon et al., 2010, 2011; Sotome et al., 2016). This class of NPs also dominates in clinical trials in dental regeneration. Novel strategies to prolong their retention in periodontal defects and to ameliorate the graft bioreactivity are under investigation (Gamal and Iacono, 2013; Al Machot et al., 2014). Similarly, NPs of amorphous calcium phosphate inhibit caries in a human *in situ* model (Melo et al., 2013). Some of the most promising nanosystems for human TE strategies are those applied in magnetic actuation, allowing for external remote control of cell responses with evident advantages for patient care (Santos et al., 2015). In summary, for the majority of herein presented NMs, the extent of the bioactivity in TE and the validation of biocompatibility have been carried out at a pre-clinical level, but randomized control clinical trials should be conducted to investigate them in humans. Available evidences indicate the importance of considering NPs for clinical practice, to be now confirmed in human studies.

Conclusions

To date, most TE studies aiming at building the essential gross morphology of organs have focused on supercellular and cellular architectures, confining research to a macroscopic level (i.e. >10 μm). However, by using nanofibers, nanopatterns and NPs to mimic the biological and structural attributes of native tissues, nanobiotechnology is clearly defining a new trend (Shafiee and Atala, 2017). The numerous applications of this principle in the literature confirm a huge potential of NPs in TE, as either agents for targeted delivery, imaging, modulation of cell functionality, biosensing, or for the structural modification of scaffolds. The opportunities offered in terms of enhanced biocompatibility and regenerative potential by TE materials derive from innovation in scaffolds' nanodesign, molecular detection and controlled delivery, as well as from cell patterning and genetic engineering of functional cell types. The present review shows that the potential impact on biomedical applications is huge and justifies that nanoscale TE will strongly develop in the near future.

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