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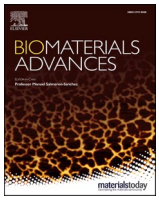

Review

Nanomaterial-based drug delivery of immunomodulatory factors for bone and cartilage tissue engineering

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ABSTRACT

As life expectancy continues to increase, so do disorders related to the musculoskeletal system. Orthopedics-related impairments remain a challenge, with nearly 325 thousand and 120 thousand deaths recorded in 2019. Musculoskeletal system, including bone and cartilage tissue, is a living system in which cells constantly interact with the immune system, which plays a key role in the tissue repair process. An alternative to bridge the gap between these two systems is exploiting nanomaterials, as they have proven to serve as delivery agents of an array of molecules, including immunomodulatory agents (anti-inflammatory drugs, cytokines), as well as having the ability to mimic tissue by their nanoscopic structure and promote tissue repair *per se*. Therefore, this review outlooks nanomaterials and immunomodulatory factors widely employed in the area of bone and cartilage tissue engineering. Emerging developments in nanomaterials for delivery of immunomodulatory agents for bone and cartilage tissue engineering applications have also been discussed. It can be concluded that latest progress in nanotechnology have enabled to design intricate systems with the ability to deliver biologically active agents, promoting tissue repair and regeneration; thus, nanomaterials studied herein have shown great potential to serve as immunomodulatory agents in the area of tissue engineering.

1. Introduction

Musculoskeletal system-related ailments, including bone, muscle or cartilage affections, are among the current concerns, especially, now that the population is aging. As a proof of concept, Liu S. et al. studied thoroughly the global burden of these motion system conditions between 1990 and 2019. Authors reported that the global burden of such diseases increased over that time period. In fact, in 2019 the incidence of detected musculoskeletal related impairments was 322.75 millions, together with 117.54 thousand of deaths, among others. The issue lies in the fact that they many times have not been given the necessary attention and can result in side effects [1].

Nonetheless, technological advances are paving the way to facilitate the diagnosis and monitoring of such impairing diseases, as well as to improve surgical interventions through real-time control of instruments. The latter is becoming necessary in bone-related operations (*e.g.*, spine or dental surgery or orthognathic repair), where incisions are performed by surgical navigation approach that enables tracking the location of surgical instruments during the surgery. These advances have also launched approaches to design tissue models for potential future biomedical robotic applications [2–4]. As instance, soft tissue modeling is enabling to develop computational scientific models that could improve tissue target-models and get haptic or three dimensional sensory experience [3,5,6].

Along these lines, this medical need to address musculoskeletal related disorders, tissue engineering has been envisioned as an

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alternative (Fig. 1). This interdisciplinary field entails the development of biological substitutes to replace, support and repair the functions of tissues by blending engineering and life science. Briefly, it comprises the construction of scaffolding systems to implant cells within the structure or assist cells attraction for tissue regeneration [7].

With regard to the musculoskeletal system, a wide array of systems have been developed –polymer-based 3D scaffolds, injectable hydrogels, among others- and exploited vast biomaterials from natural-based (alginate, hyaluronic acid, gelatin) to synthetic (polyvinyl alcohol, polyvinylpyrrolidone) to address these defects [8–17].

Nonetheless, the challenge in the field of orthopedics still remains, particularly, in bone tissue engineering (BTE), in designing constructs capable of withstanding load-bearing stresses such as that of bone tissue. A noteworthy instance is gelatin, which exhibits limited mechanical properties for BTE; therefore, it is often combined with other materials, mostly inorganic (hydroxyapatite, calcium sulfate…), given the mechanical stiffness they provide and the similarity to native tissue [18–20]. Since mechanical properties play an important role not only in bearing high pressures, but also in cell behavior (*i.e.* cell adhesion, proliferation), a recent study led by Yang K has attempted to describe the mechanisms involved in silk fibroin mechanical properties and possible stiffen approaches for future development of novel bioinspired systems [21]. Engineering scaffolds by 3D printing have also its value in assessing tissue regeneration.

Although abovementioned developments have opened up new avenues of therapy, the regeneration process of orthopedics tissue turns out to be a very complex process involving several systems. Thus, musculoskeletal system, comprising bone and cartilage tissues, is a dynamic system in which osseous or chondrogenic cells interact with immune cells (*e.g.* lymphocytes, dendritic cells, neutrophils). In this regard, osteoclasts have a close relationship with B and regulatory T lymphocytes, since these cells can activate osteoclast maturation system (RANKL/RANK system) and promote bone resorption. Likewise, cells responsible for bone formation, osteoblasts, also interact with immune cells. For example, regulatory factors secreted by T cells (*i.e.* interferon-g (IFN- γ), interleukin-17 (IL-17)) as well as the pro-inflammatory cytokine CCL2, promote differentiation towards this bone cell type [22].

Cartilaginous tissue, on the other hand, is an avascular tissue and has no regenerative capacity. The only cell type that can be found in it is chondrocytes and it has been described that these cells are able to reduce the immune response, in particular lymphocyte activity [23]. Macrophages, however, downregulate the differentiation of cartilaginous cells, and T lymphocytes can secrete factors that promote chondrocyte maturation, such as transforming growth factor-β or interleukin-3 (TGF-β, IL-3, respectively) [22].

Thus, the immune system is crucial for the adequate performance of bone-cartilaginous tissues, which explains its importance in osteopathologies and, subsequently, in the healing process [22–26]. For instance, in rheumatoid arthritis, T cells (Th17) produce an increase in the chemical compound IL-17, which promotes inflammation and osteoclast formation, among others [27]. By contrast, in the case of osteoarthritis (OA), macrophages also play an important role secreting IL-6 to enhance inflammation [27]. Regarding the healing process, M2-type macrophages have demonstrated to promote tissue regeneration,

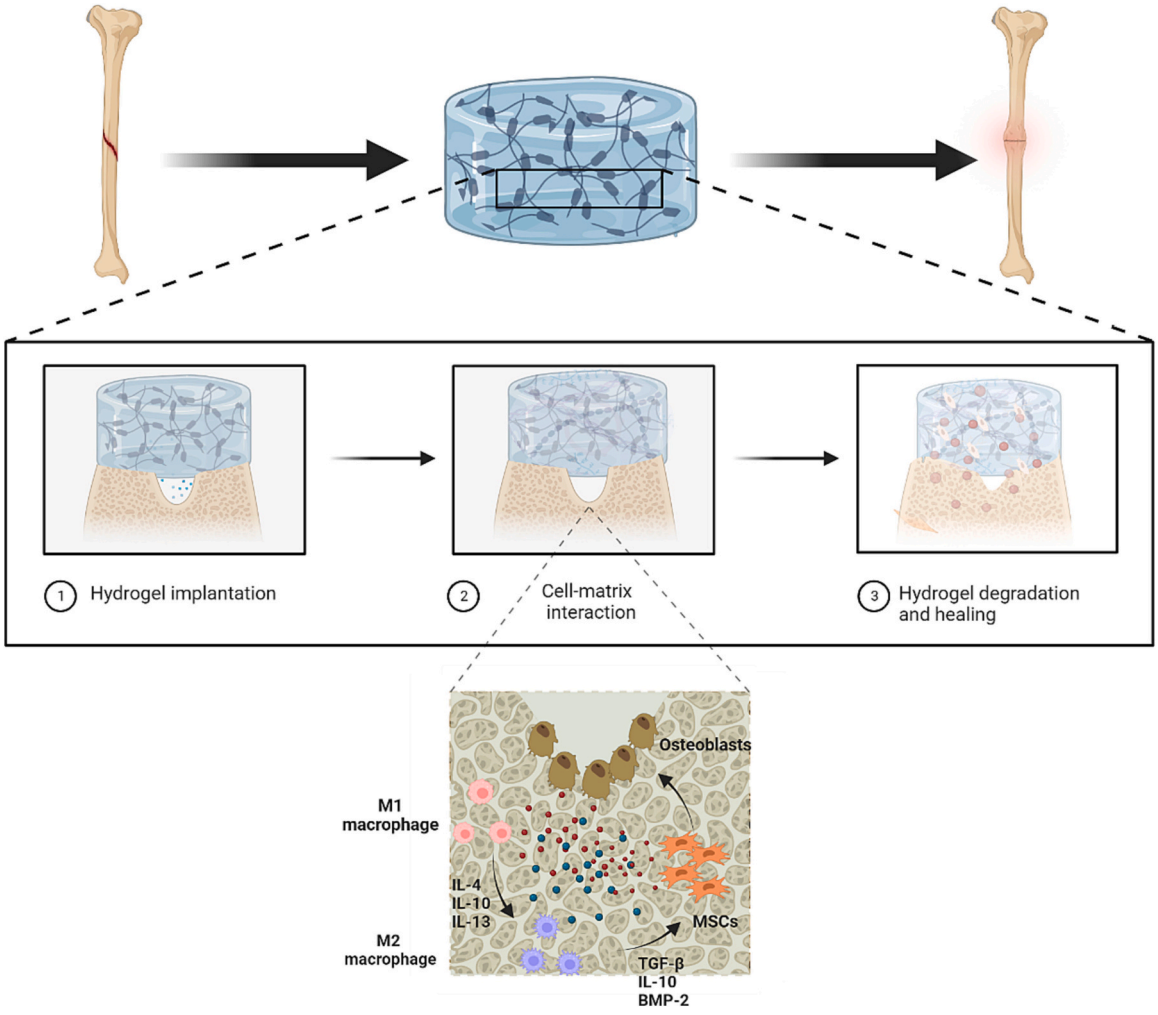

Fig. 1. Bone tissue engineering flowchart.

mediated by interleukin-10 (IL-10), TGF-β or bone morphogenetic protein 2 (BMP-2) (Fig. 2a) [28].

Given the impact of the immune system within the musculoskeletal system, emerging techniques in bone and cartilage tissue engineering (CTE) are focused on immunomodulation, *i.e.*, harnessing the immune system to generate a favorable milieu that promotes tissue regeneration [29,30].

Therefore, one of the approaches to accomplish this environment involves using immunomodulatory factors. The latter are agents that modulate positively or negatively the immune system. Some factors are cytokines (IL-4, IFN-γ), chemokines or drugs (non steroidal anti-inflammatory drugs) which have been used in immune-related diseases [31]. Given the role of the immune system in tissue regeneration, however, it is becoming increasingly important.

Within this framework, the launch of nanotechnology has bridged the gap to explore these factors in tissue engineering. Taking nanomaterials as a basis, nanotechnology can create both drug or molecule delivery systems or even facilitate tissue regeneration by mimicking native milieu [13,32–35]. Firstly, it is known that micro- and nano-environmental signals from biomaterials can alter cell-biomaterial interactions [36], and therefore resembling the natural architecture of bone using nanomaterials can be a key choice to improve tissue regeneration [33]. Secondly, nanomaterials have shown great potential as delivery platforms, enabling drugs to reach the target site avoiding systemic side effects and reducing administered doses [34,37]. What happens when using nanomaterials to target immunomodulation? Until now, it was reported that nanomaterials can produce immunosuppressant effect of immune stimulation effects [38]. For example, cerium oxide nanoparticles possess anti-inflammatory effect over murine macrophages, while silica nanomaterials present a size and surface dependent effect over THP-1 cells [39–42]. Composition, size, surface, topography and porosity of nano- and biomaterials plays an important role in immunomodulation [4,36], on this wise nanomaterials could be an appropriate tool for biomaterial-mediated immunomodulation approaches in bone and cartilage regeneration.

In light of the adequate properties of nanomaterials for musculoskeletal tissue engineering, scientists have consistently employed them. Therefore, current review will focus on the latest advances in nanomaterials as delivery systems for immunomodulation in BTE and CTE. A thorough description of nanomaterials and immnumodulatory factors used in musculoskeletal tissue engineering will be provided.

2. Immune system and tissue regeneration

As mentioned above, immune system is integrated into the musculoskeletal system and plays a very important role in repairing and regenerating damaged tissue. Specifically, immune system will be crucial in the inflammatory stage as it will orchestrate the regeneration process [43] (Fig. 3a). Considering the complex process and the pool of factors involved in tissue regeneration, different systems have been

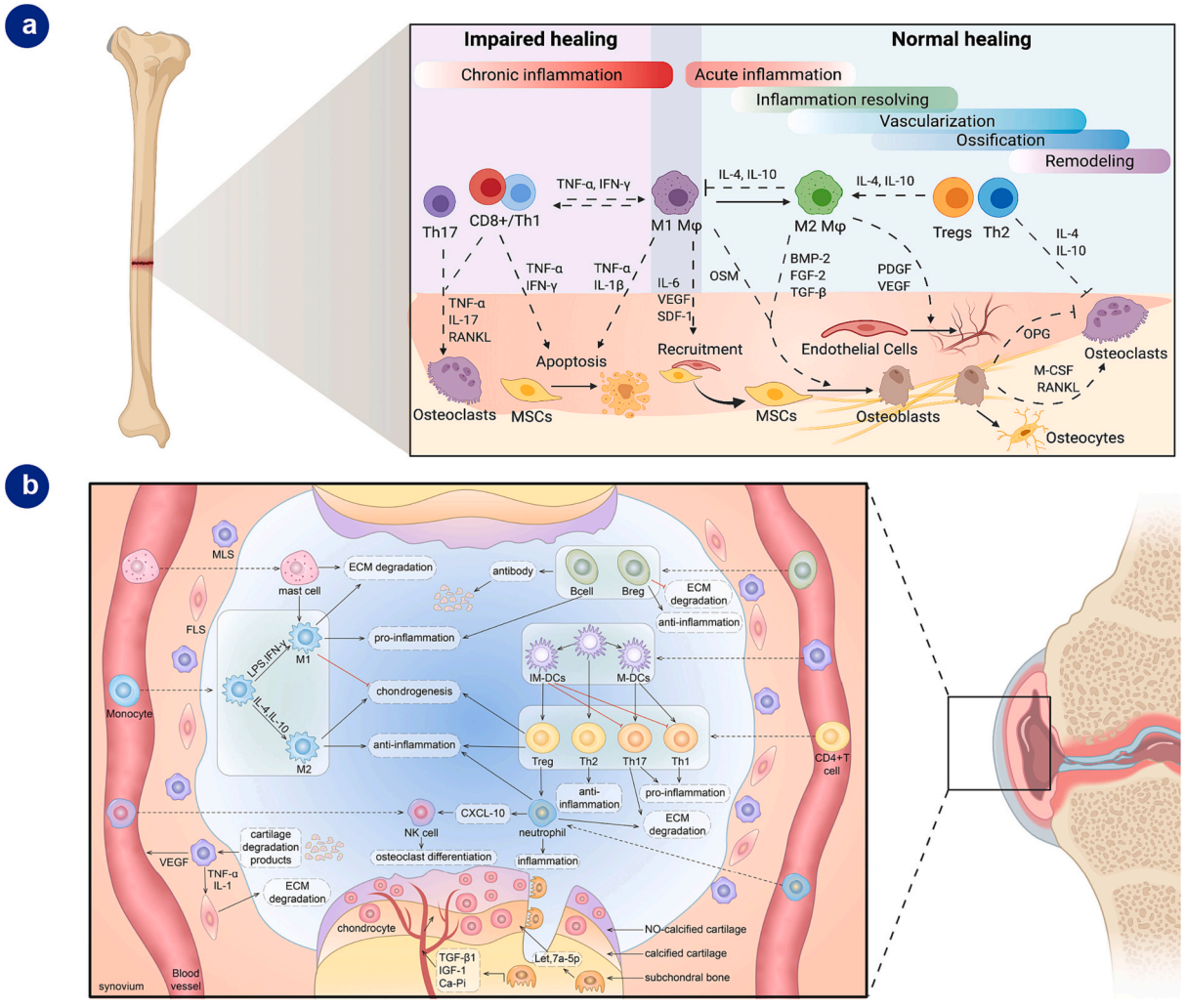

Fig. 2. Schematic illustration of musculoskeletal-immune system interaction. a) Bone healing process and immune system implication. Reproduced with permission from [25]. b) Cartilage tissue healing mechanism. Reproduced with permission from [26].

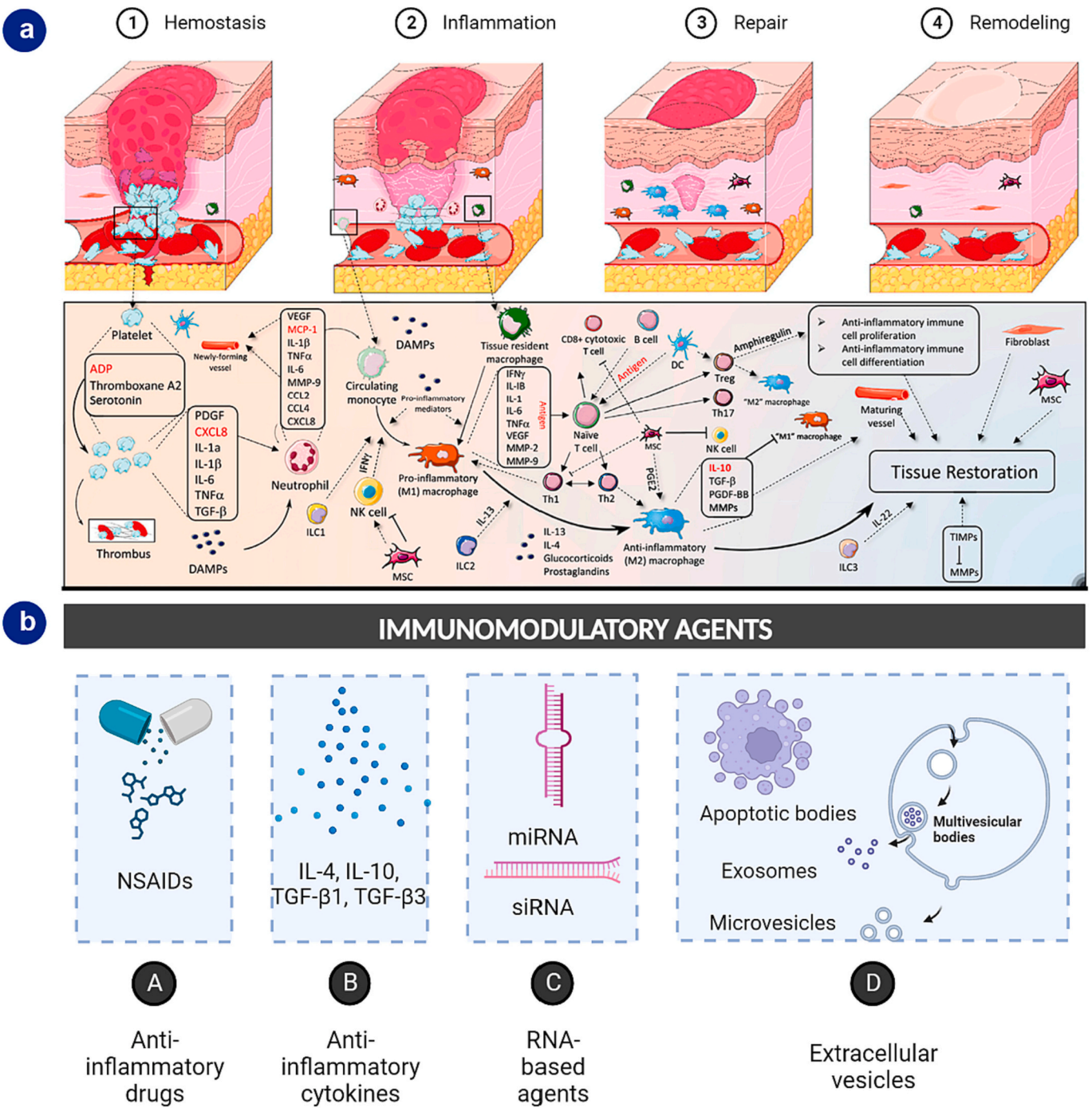

Fig. 3. Schematic illustration of tissue regeneration and immunomodulatory agents. a) Process of tissue regeneration: Hemostasis, inflammation, repair and remodeling. Reproduced with permission from [43]. b) Biological agents of immunomodulation: anti-inflammatory drugs and cytokines, RNA-based factors and extracellular vesicles.

devised to regulate inflammation by means of pro-inflammatory (*i.e.* SDF-1, PGE-2) or anti-inflammatory factors [44] (Fig. 3b). Among anti-inflammatory agents are cytokines (IL-4, IL-10) that will support macrophage migration to M2 anti-inflammatory population [44,45]. Thus, by a recently constructed system, they proved that the system led to osteogenic differentiation of MSCs by M2 polarization of macrophages and consequent release of IL-4, since antibody treatment against IL-4 anti-inflammatory cytokine reduced osteogenic differentiation of MSCs [45].

Additional biological molecules studied to immunomodulate regeneration are small interfering RNA (siRNA), microRNA (miRNA) or extracellular vesicles (EVs). In the case of the first one, siRNA, it is based on gene silencing, a very interesting therapeutic approach to regulate genes related to inflammatory cytokines, such as TNF-a [46]. The latter is a regulatory agent to activate NF-kB pathway, inhibiting osteogenesis and promoting bone resorption [47]. As for miRNA, they play pivotal biological functions: polarization of macrophages and activation of Tregs, among others [44]. Similarly, EVs are remain present today. This group comprises exosomes (EXOs), microvesicles or apoptotic bodies and can be secreted by all cells of the organism with diverse compounds (cytokines, mRNA, antigens or DNA). In cartilage regeneration, MSC-derived EXOs inhibit M1 macrophage infiltration resulting in

chondrocyte proliferation, ECM synthesis or M2 polarization; decreasing expression of inflammatory cytokines such as IL-1β or TNF-a leads to tissue repair [26]. In addition to having this immunomodulatory potential, they have the ability to act as carriers and transport molecules such as miRNA to specific sites and hence further support the treatment of the disease [48]. However, EVs limited availability in circulating tissue requires them to be blended with engineered systems such as hydrogels [49–51].

In short, immune system is permanently interacting with musculoskeletal tissue; thus, being able to exert control over this system can lead to tissue regeneration an as it is a very complex system, various potential targets can be considered, ranging from anti-inflammatory cytokines to cellular parts with immunomodulatory capacity (*i.e.* EVs). It is therefore a promising therapeutical approach in BTE and CTE to design systems that can target or at least regulate immune response. Yet, exact mechanisms underlying the roles of the immune system in tissue regeneration are not yet fully elucidated and require further in-depth studies.

3. Nanomaterials in tissue engineering

Considering the broad spectrum of nanomaterials, this section will point out several advantages, disadvantages and limitations of these materials and highlight the frequently employed nanomaterials and applications in BTE and CTE.

Nanomaterials are defined as nano-size (<1 μm) structures intended to replace or ameliorate the performance of constructed tissues or organs, due to their multifunctional ability, nanomaterials are also classified as smart biomaterials ranging from nanoparticles, nanofibers or nanotubes. Unlike macroscale biomaterials, they exhibit superior properties in terms of mechanical, physical or biocompatibility. Additionally, considering that cellular interactions take place in natural extracellular environment – a hierarchical arrangement at nanoscale-, nano-materials can promote cellular functionality (migration, adhesion, proliferation or differentiation), resulting in a promising alternative to simulate native tissue. To obtain that, parameters such as topography or increased surface area of engineered nanomaterial have been addressed [52]. As a result, nanomaterials have been widely used in engineering of diverse tissues: skin, neural or bone [53,54].

The launch of these nanometer-sized materials in BTE has allowed to obtain engineered systems with better conductive and mechanical properties, drug delivery ability or physically tailored constructs (surface, shape, size-modified) and thus promote bone regeneration. As an instance, hydroxyapatite or metal- derived nanoparticles (Au, Ag) have been widely employed to design bone-tissue like constructs with appropriate mechanical properties, whereas addition of carbon-based nanomaterials have resulted in more conductive systems [55–58].

However, the inconveniences of implanting non-degradable materials such as inorganic or carbon nanotubes in living systems remain very present today as they have triggered diverse consequences such as immune response or inflammation, resulting in failure of tissue regeneration. Extended degradation rates can also lead to long-term toxicity, yet to be clearly described [59–61]. Albeit the benefits provided by these nanomaterial-based systems are promising, it has to bear in mind that the lack of information on long-term toxicity or immune response involved in their application may hinder the translation to clinics. On the lookout of greening the nanotechnology, new alternatives are tempted to engineer nanoparticles. Algae is one of the promising candidates to reduce the exploitation of metallic-particles in designing nanoparticles, as it is economical, ecofriendly, high efficiency and there is a wide range of species (*Chlorophyceae*, *Cyanophyceae*, among others). Thus, they have been exploited in several biomedical applications such as imaging, drug delivery or biosensor [62].

Notwithstanding the inherent shortcomings of nanomaterials, nanotechnology is one of the advances that holds great promise in tissue engineering. In this line, BTE and CTE have taken advantage of an array of composites such as nanoparticles, carbon-derived materials or transition metal dichalcogenides that are following described.

The launch of nanoparticles in orthopedics provides convenient platforms to encapsulate a series of compounds ranging from growth factors (BMP-2, VEGF, TGF-β1) to antimicrobials and maintain their administration over time, resulting in stimulation of tissue regeneration [63–65]. These nanoparticles have been composed of metallic materials such as gold, silver or aluminum, which in addition to their encapsulation capacity, have shown to possess the inherent ability to promote cell proliferation, inhibit antibacterial activity or improve mechanical properties [66]. Mesoporous silica nanoparticles (MSN) have also gained importance in the musculoskeletal field, since silicon is an abundant element in both bone and cartilage. Silicon can stimulate the proliferation and differentiation of bone forming cells (*i.e.*: osteoblast) playing an important role in osseointegration and bone growth. In this sense, silica-derived systems have proven to be effective as drug delivery platforms, as well as having bioactivity. Specifically, the gradual release of Si ions promotes tissue regeneration [67].

Within nanomaterials, carbon-derived two-dimensional (2D) materials have likewise been widely used in BTE and CTE, particularly, graphene oxide (GO) and reduced GO [68–70]. In a recent study, Li et al. demonstrated a GO-based system as a potential therapeutic for OA, as it was able to release in response of light an anti-inflammatory agent over time. In addition, the inherent mechanical strength of graphene allowed the bearing stress to be dispersed, and a lubricated environment could be ensured for a long period [71]. Besides the latter, other scaffolding systems have demonstrated the potential to improve the mechanical properties of the design by adding this carbon derivative [72]. Its surface functional groups too enable these compounds to interact with other materials [73–75]. Thus, this nanomaterial is either combined with other materials to form systems that promote bone tissue regeneration or with biological agents to serve as drug delivery systems [71,72,76,77]. For instance, GO has been combined with BMP-2 to ensure a controlled release of this pro-osteogenic agent in bone regeneration or TGF-β for cartilage repair [76,77]. GO has also been blended with immunomodulatory factors such as IL-4 to intervene in the process of macrophage polarization and thus promote tissue regeneration [78].

Apart from carbon-derived materials, transition metal dichalcogenides (*i.e.* MoS_2) have been employed. This material can produce a photothermal effect that induces oxidative or antibacterial activity in the tissue regeneration process. These effects have been observed upon application of this nanomaterial under near infrared light (NIR). In musculoskeletal impairment such as OA, MoS_2 has acted as an on-demand delivery platform, in which the release of the drug is controlled by NIR light. The latter provides control over the time as well as the site of administration, which can result in a reduction of the corticosteroid dose [79–81].

Additional two-dimensional compounds that are gaining attention lately in BTE and CTE are clay nanoparticles (*i.e.* Laponite ® RD, Montmorillonite). These are nanosized silicate derivatives with a tetrahedral-dioctahedral-tetrahedral structure. Such intricate multilayer architecture allows these nanomaterials to have properties such as high specific surface area or cation exchange capacity, allowing interactions with other compounds. Together with that, they are biocompatible and have good swelling ability. When it comes to supporting tissue regeneration, their inorganic nature enhances the inherent mechanical properties of polymeric hydrogel systems to simulate the mechanical characteristics of load-bearing tissues. In addition, these nanoparticles have been shown to promote the differentiation of mesenchymal stem cells (MSCs) into osteoblasts and have the ability to act as delivery systems for bioactive agents (BMP-2, SDF-1) to further support tissue regeneration [13,82,83]. As a proof of principle, in a study conducted by Erezuma demonstrated that nanoclay-reinforced composite hydrogel had the ability to differentiate MSCs into osteoblast within 3 weeks as well as the capability to deliver biologically active *agents in vivo* (SDF-1 and cells) resulting in enhanced tissue regeneration [13].

As discussed so far, nanoparticles can serve to regenerate tissues such

as bone or cartilage or as delivery platforms, but they can also exert an immunomodulatory effect *per se*. Another emerging application has been derived as a result of nanomaterials ability to regulate immune system, since by their very nature can have immunomodulatory effects that enhance tissue regeneration [84]. In this regard, it is reported that MSN can have an impact on immune system. Particularly, In a study carried out by Chen et al., it was observed that MSNs, when engulfed by macrophages, were able to inhibit the Wnt5A/Ca^{2+}pathway, which is responsible of activating the transcription factor nuclear factor kB (NF-kB) [67,84]. Authors demonstrated that the engineered system had the ability to achieve an anti-inflammatory effect while promoting osteogenic differentiation which goes in accordance with other studies where the role of MSN in the regeneration of bone tissue have been described [67,84–86]. Another noteworthy study, however, evaluated the capacity of nanohydroxyapatite particles as a platform to interface with immune system. *In vitro* and *in vivo* tests concluded that nanohydroxyapatite particles could influence M2 macrophage polarization and IL-10 anti-inflammatory cytokine secretion [87].

In the attempt to modify surface topography, Zhang and colleagues investigated the immunomodulatory osteogenic effects of micro- and nano-structured titanium dioxide (TiO_2) and zinc oxide (ZnO) coatings on pure titanium (Ti) [88]. In a previous approach, researchers utilized hydrothermal and heat treatments to create a TiO_2/ZnO coating with granular nanostructures, and had proven its osteogenic ability [89]. In this study, instead, researches went a step further and examined the effect of the coating in immunomodulation. It showed that the nano-structured coating polarized M1 and M2 macrophages and increased osteogenesis compared to macroporous TiO_2, and thereby it can be concluded that the system has potential as a dental implant coating material [88]. However, in a work done by Cockerill et al., they engineered Zn plates with nano-, submicro-, and microtopographies. The latter displayed an increased alkaline phosphatase (ALP) activity, enhanced COL1A and Osx gene expressions and less M1 polarization than the nanotopography plate [88].

As a conclusion, the launch of nanotechnology have paved the way to design materials, such as nanomaterials, that can further enhance tissue regeneration by virtue of nanometric size, or by their interaction with the immune system. Their properties will permit to mimic cell environment even closely, resulting in improved tissue healing; thus, application and study of such materials in bone tissue and cartilage engineering is very extensive. Albeit alternatives – use of naturally derived nanoparticles- are already being sought, adverse-effects (long-term toxicity or regulatory issues) are still a challenge and still need to be studied.

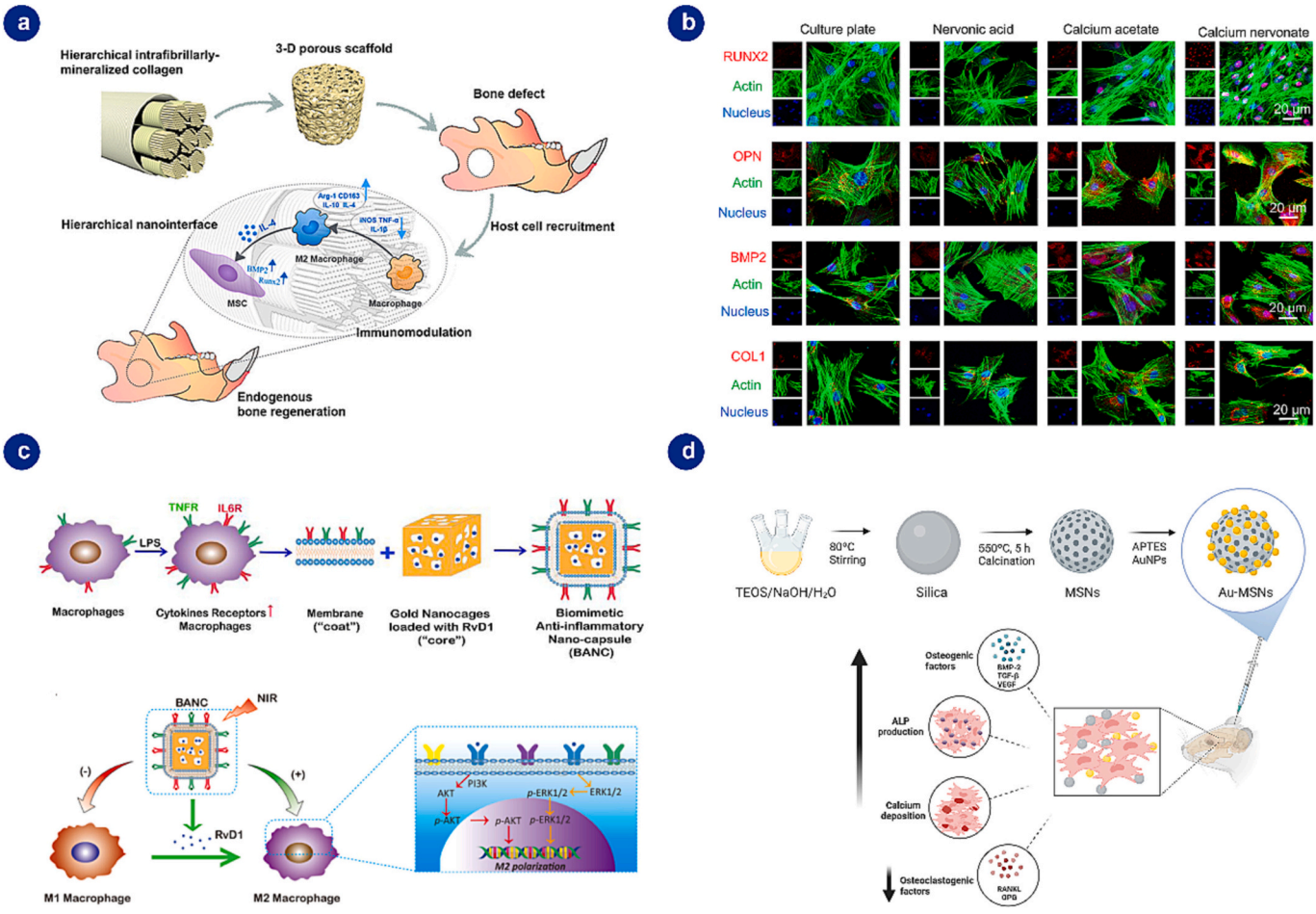

Fig. 4. Nanomaterial-based systems for bone tissue engineering. (a) Schematic illustration of a hierarchical intrafibrillar mineralized collagen with a bone-like nanostructure. Reprinted with permission from [45]. Copyright {2019} American Chemical Society. (b) Immunofluorescence staining results of mesenchymal stem cells. Cells were treated with 0.05 mM nervonic acid, calcium acetate or calcium nervonate nanoparticles. Red staining corresponds to osteogenic markers (RUNX2, OPN, BMP-2 and COL1). Phalloidin stained F-actin green and nuclei were stained blue by DAPI. Reproduced with permission from [97]. (c) Illustrative diagram of the design of the loaded gold nanocapsules and their effect on macrophages. Reproduced with permission from [108]. (d) Synthetic pathway diagram of Au-MSNs and regeneration process of the cranial defect.

4. Recent advances on nanomaterial-based immunomodulatory factors delivery

4.1. Bone tissue

On the lookout of personalized medicine, osteoimmunomodulation, namely, immune regulation for bone regeneration purposes, is one of the field that is gaining importance in BTE. In this vein, nanotechnology might be considered a paradigm shift to engineer systems to regulate immune response. Nanomaterials, particularly, may play an important role in the immune system since they can regulate immune system *per se* thanks to nanotopographies, but also acting as delivery platforms for immunomodulation. Nanostructures have suitable properties to provide a controlled site-specific delivery of myriad molecules –drugs, cytokines and growth factors– and for different purposes [34]. Over the last few years, several works have been published in this regard.

For instance, Jin and colleagues in an attempt to better mimic bone complex structure, created a hierarchical intrafibrillarly-mineralized collagen scaffold with a bone-like staggered nanointerface (HIMC) to promote bone regeneration (Fig. 4a) [45]. Once the scaffold demonstrated MSCs recruitment and differentiation *in vitro* by M2 macrophage polarization, the authors implanted the system embedded with M2 macrophages released IL-4 cytokine into criticalsized defects in rat mandibles. Furthermore, the impairing bone formation by monocyte/macrophage depletion was also assessed. The construct allowed to elucidate the mechanism of immune system in bone regeneration process as it was concluded that bone regeneration could happen by the ability of the designed system to polarize macrophages to secrete IL-4 and subsequent inhibition of monocyte/macrophage depletion [45].

By the same token, Li et al. exploited nanotechnology to devise a system to address one of the most prevalent titanium implant-related issues (implant associated infection). The advances on nanomaterials permitted to design a programmed local delivery system based on a micro-nano surface of Ti [90]. Ti surface was heat-treated and *a posteriori* combined with poly-dopamine (PDA) to construct AH-Sr-AgNP structure with the ability to release Ag^{+} and Sr^{2+}. Both *in vitro* and *in vivo* studies evidenced that the sustained release of such elements enabled antibacterial activity as well as immunomodulation *via* M2 macrophage polarization, resulting in osteogenesis [90]. In a follow-up work TiO_2 nanotubes were studied as immunomodulatory agent system. IL-4 was loaded with PDA and a carboxymethyl chitosan hydrogel layer, which controlled cytokine liberation and set up RGD peptide immobilization into TiO_2 nanotubes [91]. The authors used an indirect co-culture method to analyze the impact of both osteogenic efficacy of material-induced MSCs and material-mediated immune microenvironment. A synergetic effect was observed between the immunomodulation achieve by the system (macrophage polarization) and the osteoinductive biomaterial, which enabled osteogenesis [91]. In another study led by Wang et al., Ti nanotube arrays were molded for the delivery of macrophage-derived EXOs. This was the first attempt to observe the immunomodulatory and angiogenic capacity of these systems. By culturing the EXOs delivery systems with mesenchymal and endothelial cells, findings indicated that EXOs promoted angiogenesis in endothelial cells, as well as activated markers of osteogenesis in mesenchymal cells, thus enabling bone regeneration. However, the underlying mechanisms involving macrophage-derived vesicles in immunomodulation could not be elucidated [50]. In other studies, researches incorporated elements such as Zn or Ag to TiO_2 nanotubes in order to stimulate M2 macrophage polarization and to promote osteogenesis [92,93].

Another ion that has gained attention in bone regeneration is calcium, since 70 % of bone consists of calcium phosphate [94,95]. Therefore, progress in nanomaterials have enabled to design nanoparticles that enable the delivery of this element [96,97]. As an instance, Ma et al. designed calcium nervonate-based nanoparticles. These nanosized particles were uptaken by cells and degraded by lysosomes. As a result, nanoparticles were cleaved into calcium ions and nervonic acid. On the one hand, calcium had favorable osteogenic response in MSCs (Fig. 4b). On the other hand, immunity studies with M1-induced macrophages showed that both calcium nervonate- and nervonic acid-treated macrophages had reduced gene expression of pro-inflammatory factors (IL-6, IL-1β). All these effects were further confirmed by *in vivo* studies. The system could support bone regeneration, by providing an additional osteogenic and anti-inflammatory environment [97]. Zhang et al., additionally, sought to shed light on the immunomodulatory role of calcium ion in bone regeneration. On the basis of the role of macrophages in tissue regeneration, they conducted a study with macrophage wild-type animals and found that osteoinductive capacity was higher in comparison to macrophage-deprived group. Therefore, it was concluded that calcium controlled delivery can induce M2 polarization and thus, tissue regeneration [98].

Along these lines, it is worth noting that calcium-based composites have been widely employed in tissue engineering because of their native tissue-like composition. Calcium phosphate is the example of either graft or nanomaterial extensively exploited in bone regeneration [99]. In contrast to calcium phosphate derived grafts, nanoparticles provide several benefits such as higher bioactivity, controlled biological agents delivery, better integration in the tissue or easy fabrication. In addition, their high specific surface contributes to improved cell performance (adhesion, proliferation, differentiation) [99]. As a proof of principle, in a study carried out by Zhao and co-workers, anti-inflammatory cytokine (IL-4)-loaded calcium-phosphate containing system was engineered to promote bone regeneration. *In vivo* results demonstrated that IL-4-releasing structures obtained higher bone regeneration ability compared to pristine scaffolds. In particular, IL-4 triggered the polarization towards M2 anti-inflammatory macrophages and consequently the secretion of osteogenic protein (BMP-2) [100]. In another attempt, Wang and collaborators, designed immunomodulatory RNA (commercial RNA II) loaded calcium phosphate nanoparticles embedded in polymeric matrix for bone regeneration. Results were in agreement with abovementioned studies: RNA containing calcium composites upregulated the migration towards M2 cells and the latter released a series of anti-inflammatory factors that promoted tissue regeneration [101]. Although it is possible to observe the regulatory role of calcium in modulating the immune system and the drug delivery ability of calcium phosphate nanoparticles, and, consequently, tissue regeneration ability, yet the detailed signaling pathways remain to be fully described.

Another alternative for calcium or other ions delivery have the potential to be MSNs. Thus, a study demonstrated that europium-doped mesoporous silica nanospheres (Eu-MSNs) were a great tool for inducing osteogenesis and angiogenesis through immunomodulation [102]. Europium can act as calcium, which means that it can stimulate bone regeneration process. Researches created a conditioned-medium in contact with nanospheres and macrophages, and then they used that medium to culture both bone marrow MSCs (BMSCs) and human umbilical vein endothelial cells (HUVECs). *In vitro* results revealed enhanced osteogenesis and angiogenesis with Eu-MSNs compared to MSNs alone. Also, in the *in vivo* rat cranial defect model the authors detected more regeneration capacity in the Eu-MSN group after 12 weeks. All the results suggested that this improvement in regeneration was due to the modulated immune microenvironment created by Eu-MSNs and macrophages [102].

In the same strain, Liang et al. used MSNs loaded with gold (Au-MSNs) to enable osteogenesis *via* immunomodulation [103]. Macrophages were cultured with Au-MSNs to create an immune microenvironment –macrophage polarization, cytokine and osteogenic factor release–, which was responsible of promoting bone regeneration *in vitro* and *in vivo* (Fig. 4d*)* [103]. Guo et al. also promoted bone healing by sustained miRNA delivery from MSNs. *In vitro* studies demonstrated the role of miR-34a in inhibiting osteoclast bone resorption, which may delay fracture remodeling and enhance fracture healing [104]. In another study, MSNs served as a platform for dual delivery of antibiotic

(cefazolin) and growth factor (BMP-2) into the bone fracture. *In vitro* studies showed that the controlled release of the drug prolonged the half-life of the pharmaceutical, thereby reducing the dose required. Cells treated with the complex also proved increased osteogenic ability upon BMP-2 administration. In animal studies, this compound system could modulate inflammation, as there was a reduction of pro-inflammatory cytokines (IL-1β and IL-4) and an increase of anti-inflammatory cytokines (IL-10) [63].

Nanotechnology has also fueled the use of drug delivery systems, such as liposomes -phospholipid vesicles- to act as drug delivery agents in tissue engineering. Given the properties of these lipid structures (biocompatibility, low immune response and loading capacity), liposomes provide a well-established basis for BTE [105]. In this line, nanomaterial advances allowed to Li et al., to create three-dimensionally (3D) printed liposomes loaded with the anti-inflammatory drug aspirin (Asp@Lipo) and to deposit them in a polycaprolactone (PCL) scaffolds (PCL-Asp@Lipo) [106]. The authors demonstrated its ability to regenerate bone with different *in vitro* and *in vivo* studies, the latter manifesting that the created structure promoted immunomodulation due to a decrease in both TNF-α and IFN-γ concentrations in a subcutaneous rat model [106].

Additionally, in the search to simulate natural milieu, Yin and collaborators took advantage of nanotechnology to design cell membrane-coated nanoparticles, as the latter biological structures may provide the system with natural cytokine receptors and ligands [107]. Particularly, the aim of the study was to construct a biomimetic anti-inflammatory nano-capsule (BANC) as immunomodulation agent for bone repair [108]. The system consisted of a gold nanocage coated with lipopolysaccharide pre-treated macrophage cell membrane and loaded with RvD1 (M2 macrophage polarization inducer). The coating membrane had receptors for TNF-α and IL-6 cytokines, so these molecules bound to the structure and the overall inflammation reaction was reduced (Fig. 4c). Apart from that, *in vitro* and *in vivo* results showed that BANC was able to transport and release RvD1 factor in a precise way under NIR irradiation, which enables an enhancement on M2 macrophage polarization. What is more, to some extent the system itself was able to induce M2 migration due to its nanosize [108].

Another naturally inspiring approach to use as drug delivery systems are EXOs. As mentioned above, exosomes are nanostructures that are becoming increasingly valuable in BTE since they are natural, enable transporting of an array of compounds and enhanced cellular uptake (through surface adhesion molecules). In this line, progress in nanotechnology allowed to exploit EXOs as immunomodulatory factors delivery system [109]. A noteworthy study, have taken advantage of EXOs to load with TGFB1 and IL-10 and inhibit degenerative bone disease [110]. The authors isolated EXOs from dendritic cells, embedded them with immunoregulatory molecules and administered them both intravenously and locally to reprogram the Th17-mediated immune response with the aim of attenuate alveolar bone loss. Results showed that EXOs protected cytokines from degradation; thus, those molecules could perform their synergistic regulatory effect, and a reduction on osteoclast-mediated bone degeneration was achieved [110].

Apart from acting as delivery platforms for bone regeneration purposes, nanomaterials could also be used to treat specific diseases or to use them for gene-delivery. In such regard, progress in nanotechnology has allowed to design systems targeting specific cell groups, as it is in the case of cancer. For instance, Pang and co-workers modified the surface of cytosine-phosphate-guanosine-loaded metal organic framework nanoparticles with zoledronic acid (ZOL) to treat breast cancer bone metastases [111]. Owing to ZOL, which has ability to join bone tissue, the system could achieve a strong binding and an accumulation in the bone, and therefore it was able to realize its function in a specific way. Moreover, ZOL inhibited osteoclast production and function, an important step to cease the vicious cycle of bone metastasis. Besides, the effective delivery of immunostimulatory cytosine-phosphate-guanosine and its uptake by the cells activates TLR9 receptor and subsequently inhibited RANK-L induced osteoclastogenesis. Additionally, the created nanoparticles enhanced macrophages M1 polarization, thus forming a pro-inflammatory environment, which may reduce tumor growth [111]. In another attempt, Yin et al. engineered another therapeutic approach for osteosarcoma [112]. The system was composed of ZIF-8 nanoparticles for the delivery of the chemotherapeutic and immunosuppressive agent (methotrexate). Since these nanoparticles were modified by a photothermal agent (PDA), the antitumor effect was amplified, on the one hand, by the release of the drug, which turned out to be dependent on NIR stimulation, and on the other hand, by the ability to transform light energy into thermal energy. This phenomenon resulted in the increase of temperature, and consequently the death of the osteosarcoma cells *in vitro* [112].

For metabolic skeletal disorders such as osteoporosis, progress in nanomaterials have shown to have potential to enable gene-therapy. In that vein, Li and colleagues were based their approach on anti-inflammatory compound salicylic acid to fabricate poly(salicylic acid) nanoparticles (PSA-NPs) to deliver microRNA-21 (miR-21) as immunomodulatory agent [113]. To own miR-21 targeting ability, nanoparticles were treated with $(Asp\text{-}Ser\text{-}Ser)_6$ $(DSS)_6$ peptide. Once the system was constructed, authors proved its ability to improve bone regeneration in a mouse osteoporotic bone model. Results showed that miR-21@PSA-NP-$(DSS)_6$ significantly reduced TNF-α and IL-6 levels, both pro-inflammatory cytokines, reducing the inflammatory environment created in osteoporosis. Aside from that, the delivery of miR-21 enhanced osteogenesis as well, and it was concluded that the advances in nanotechnology permited to design a miR-21@PSA-NP-$(DSS)_6$ system that hold great promise to improve osteoporosis enhancing both anti-inflammatory effect and pro-osteogenic effect [113].

Nanomaterials have also proven to be a valuable tool in inflammatory processes stemming from surgical interventions such as implant placement, as it often results in inflammatory osteolysis, causing bone loss [114,115]. In the attempt to address the latter, Ding and co-workes relied on emerging techniques to design cell membrane-derived nanoparticles. As mentioned above, unlike other nanoparticles, these biological engineered nanomaterials are advantageous because of their biocompatibility and tissue specificity through ligand-receptor interactions [116]. Thus, in this study, SiO_2 nanospheres loaded with Se and coated with macrophage membrane (M-Se@SiO_2) (Fig. 5) were constructed [117]. Thus, *in vitro* evaluation showed that coating by a macrophage-derived membrane reduced endotoxins. The underlying mechanism relies on receptors found on the macrophage membrane (TLR4, IL6-R, TNFR) that could bind to endotoxins as well as to specific pro-inflammatory cytokines. Furthermore, Se release promoted the differentiation of anti-inflammatory macrophages (M2). All this could be further confirmed in osteogenic cell and *in vivo* studies (Fig. 5). Bone formation process was inhibited in untreated cells (LPS-stimulated group) compared to treated cells, in which osteogenic markers (ALP, alizarin red) were elevated [117].

In summary, progress in nanotechnology has allowed the design of systems ranging from nanoparticles to release elements involved in bone regeneration (*i.e.* Ca, Sr, Zn) to polymeric systems enclosed by cellular components to interface more effectively with immune systems, liposomes, EXOs, among others Accordingly, it has been feasible to engineer systems that permit the targeted delivery of immunomodulatory agents that could promote bone regeneration. Advances in this interdisciplinary field have also served as a tool to treat several bone-related diseases, such as osteoporosis or bone metastasis, in which anti-inflammatory drugs therapeutics are usually employed.

4.2. Cartilage tissue

In CTE, the challenge remains in mimicking a load-bearing yet dynamic system. Therefore, progress in technology –*i.e.* 3D or 4D printing- has bridged the gap between materials and engineering that result in development of systems with cartilage-like properties. As a proof of

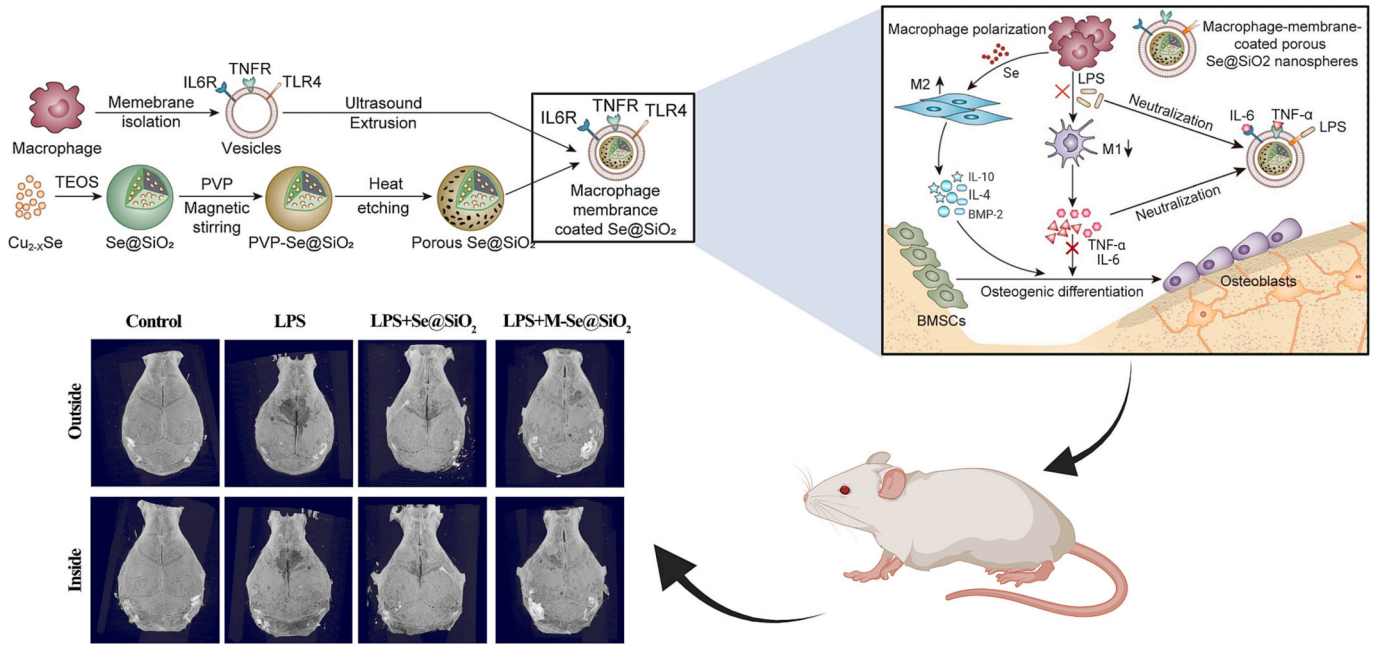

Fig. 5. Biomimetic nanomaterial-based composite for inflammatory osteolysis treatment. Flow diagram of nanocomposite (Macrophage-biomimetic porous Se@SiO_2) synthesis and mechanism of action. Bottom: 3D images of Micro-CT study where M-Se@SiO_2 mitigates LPS-induced osteolysis *in vivo*. Reproduced with permission from [117].

concept, Díaz-Payno et al. took advantage of 4D bioprinting to engineer complex curved system based on gelatin and hyaluronan. The addition of alginate to the hydrogel allowed creating stiffer constructs that could bend when immersed in aqueous solution and cell incorporation to the hydrogel resulted in multi-layered cell structure with cartilage-tissue like matrix deposition. Authors concluded that 4D bioprinting has the potential to fabricate intricate tissues, as it can mimic the shape and cell density of the native tissue [118]. As cartilage is an elastic and soft tissue, another approach that has been used in CTE is to use materials with deformation ability, but able to support native tissue mechanical properties [119–121]. A noteworthy material is the aliphatic polyester derived poly (glycerol sebacate) (PGS). This synthetic polymer is known for its good biocompatibility, low toxicity, biodegradability and elastic properties. Additionally, the composites (sebacic acid and glycerol) are natural-origin and FDA approved [122]. In contrast, the crosslinking conditions are harsh (temperature above 120 °C) and curing time is long.

Nanotechnology enabled to address this issue, combining PGS with inorganic nanomaterials such as hydroxyapatite or nanoclay. By this means, it has been possible to design systems with superior electrical and mechanical properties, as well as thermal stability [120,121]. In this line, a recent study leaded by Asgharnejad-laskoukalayeh engineered a PGS-based system with nanohyroxyapatite. The use of nanotechnology permitted to design thermally stable composites with controlled degradation and enhanced biocompatibility and cell adhesion [120].

As in the case of bone, nanomaterials also offer suitable properties to act as drug-delivery platforms for CTE purposes [123]. Latest breakthroughs in nanotechnology have facilitated that various immunomodulatory factors or drugs could be attached to these nanometer-sized materials to ease their transport and drug delivery.

For example, numerous studies had used nanomaterials to release transforming growth factor-β3 (TGF-β3), a potent immunomodulatory agent that promotes cartilage regeneration [124–126]. The latter mentioned factor has a huge drawback: it has a very short half-life (<30 min) as it is degraded by the body enzymes, and therefore new methods for carrying and releasing the growth factor in a precise way are still needed. Novel approaches in nanotechnology have emerged as an alternative to overcome these difficulties. In that instance, Qu and co-workers used polycaprolactone (PCL) and polylactide-*co*-glycolide (PCL-PLGA) nanofiber scaffolds to release TGF-β3 in a proper manner to achieve fibrocartilage regeneration (Fig. 6a) [124]. Exploring nanofibers as a delivery platform for TGF- β3 permitted, on the one hand, to simulate cartilage intricate structure and, on the other hand, a controlled release and a better interaction with cells as a result of the high specific surface area of nanofibers. Specifically, the authors showed that a burst-release occurred within the first 24 h, while the release kinetics changed to a sustained one afterwards. The investigators concluded that the designed system was effective, since it created fibrocartilaginous matrix due to the local administration of the growth factors [124]. In another study, sustained release of TGF-β3 was obtained by three different nanoparticulated systems, consisting of (I) high-acetylated chitosan CS [42] and tripolyphosphate (TPP) (CS(42)-TPP), (II) low-acetylated chitosan CS [17] and TPP (CS(17)-TPP), and (III) alginate (Alg) and poly-L-lysine (PLL) (Alg-PLL). The nanosystems enabled high encapsulation efficiency and release sufficient TGF-β3 to promote chondrogenesis; however, chitosan-based ones exhibited more ability to induce differentiation although their reduced efficiency to release TGF-β3 compared to alginate ones [125]. Another alternative to deliver TGF-β3 recently developed by Barati and colleagues involved engineering PDA-coated MSNs to achieve sustained release of this growth factor from a macroporous gelatin microribbon scaffold [126]. The high-surface area of MSNs and the slow degradation of PDA-coating were the responsible of the sustained release of the growth factor, which ended up with a continuous cartilage deposition in an *in vivo* subcutaneous model [126].

In another vein, there are also other factors implicated in the cartilage tissue physiopathology, including TNF-α. Thus, various studies have focused their efforts fabricating nano-systems to deliver sex-determining region Y-box 9 (SOX-9) plasmid or silenced TNF-α (siTNFα), since it has been observed that TNF-α can down-regulate SOX-9, which activates inflammation and restricts chondrogenesis [127]. One of the latest approaches reported the use of carbon-based nanomaterials- dual-functional cationic carbon dots (CDs) - to deliver SOX9 plasmid to induce chondrogenesis. This system enabled efficient gene transfection as well as intracellular monitoring of administered molecules *via* fluorescence, and SOX9 administration demonstrated

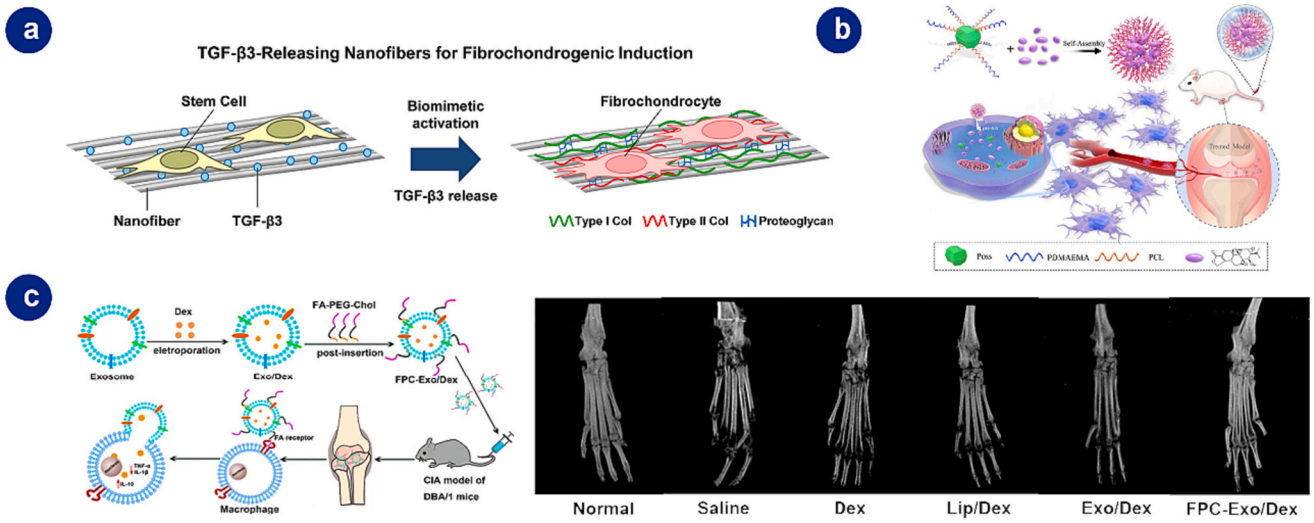

Fig. 6. Illustrations of cartilage tissue engineering *via* immunomodulation. (a) Graphical illustration of the process of fibrochondrogenic differentiation through the release of transforming growth factor-β3 from nanofibers. Reproduced with permission from [124]. (b) Scheme of the designed construct loaded with low doses of the anti-inflammatory agent triptolide and pH sensitive. Reproduced with permission from [133]. (c) Engineering approach of exosomes-based nanoparticles and representative images of mouse hind limbs reconstructed in three dimension from micro-CT analysis. Reproduced with permission from [139].

chondrogenesis [128]. Along with this, Lio et al. also employed carbon dots, but with the aim to deliver siTNFα, to silence mRNA of TNF-α and thus promote chondrogenesis [129]. The designed platform showed potential for chondrogenesis *in vivo* due to its ability to reduce TNF-α levels [129]. In another attempt, cationic nanosized liposomes were used to transfect rat bone marrow stromal stem cells (rMSCs) with SOX-9 [130]. Once cells were transfected, thermos-sensitive chitosan hydrogels were embedded with cells and the created systems were injected subcutaneously in a nude mouse *in vivo* model. Results showed that liposomes were an excellent tool to achieve a good gene delivery and consecutive enhanced tissue regeneration [130].

Progress in nanotechnology has also paved the way to cartilage-related impairments, such as rheumatoid arthritis or osteoarthritis. Specifically, nanomaterials hold great promise as drug delivery platforms for an array of immunomodulatory agents (*i.e.* immunosuppressive or anti-inflammatory drugs).

Rheumatoid arthritis is a systemic autoimmune disorder, characterized by inflammation, pain, stiffness, and loss of joint function; its treatment is often difficult due to, among other things, the adverse effects produced by unspecific binding of drugs in the tissues [37,131]. Several studies have been fact-finding how to carry and release drugs in a more precise way by using nanomaterials. For example, Zhang and coworkers loaded triptolide (TP) –an immunosuppressive and cartilage-protective molecule– into poly-γ-glutamic acid-grafted Asp (PA) nanocarrier [132]. The created system enabled a slow release of the drug, which decreased the drawbacks and allowed a sustained efficacy of TP. More recently, researchers have combined pH-responsive poly(methylaminoethyl methacrylate) (PDMAEMA) with PCL to generate nanoparticles capable of transporting TP (Fig. 6b) [133]. On this occasion as well, nanoparticles showed efficacy to reduce systemic side-effects of TP, as well as a great anti-inflammatory effects even with low dosages [133]. Hu et al. went a step further and designed a dual anti-inflammatory system by the controlled release of indomethacin (non-steroidal anti-inflammatory drug) and the photothermal activity of the nanomaterial (Prussian blue nanoparticles). As an extra incentive, the complex was encapsulated with a membrane (obtained from red blood cells and macrophages) that was capable of recognizing CD44 (an abundant receptor in inflammatory milieu). As a result of these properties, *in vitro* assays demonstrated their ability to adhere to the moist environment of the joint. *In vivo* studies served to confirm the capacity of these nanomaterials to target macrophages in joint tissue and thereby to provide an anti-inflammatory environment by promoting apoptosis and reducing the secretion of pro-inflammatory cytokines [134].

Following a similar line, emerging techniques permitted to manufacture nano-platforms for other drugs and molecules such as methotrexate (MTX), dexamethasone (Dex) or 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1). Lyu and colleagues used mannose-modified serum albumin nanoparticles as vehicles to carry and release MTX (MTX-M-NPs) [135]. Nanoparticles allowed a sustained and local release of the drug, which ended with less side-effects and a better therapeutic dosage of MTX [135]. Pentecost et al. fabricated dexamethasone-loaded octadecylamine-functionalized nanodiamonds (ND-ODA-Dex) for rheumatoid arthritis treatment. *In vitro* assays showed the potential of the system to act as delivery platforms, but the authors concluded that deeper *in vivo* studies are needed to determine the effectiveness of ND-ODA-Dex [136]. Likewise, Wang et al. made use of gold nanocages to achieve a local delivery and low dosage of TPCA-1 –nuclear factor-κ pathway inhibitor– to treat rheumatoid arthritis [137]. Aldayel and collaborators employ lipid nanoparticles for TNF-α siRNA delivery [138]. Nanoparticles were composed of lecithin, cholesterol, and stearic acid-polyethylene glycol (2000) hydrazone conjugate (PHC), which endowed it with an acid-sensitive property. Results showed that these nanosized particles had the ability to avoid a burst-release and enable an acceptable anti-inflammatory therapy [138].

In the quest to reduce the use of exogenous materials, EXOs have also gained prominence in rheumatoid arthritis therapy. This endogenous nanosized structure facilitates specific and prolonged drug release. As a proof of concept, Yan and colleagues used macrophage-derived EXOs as release systems for dexamethasone. For even more selective and sustained delivery, EXOs were functionalized with folic acid, PEG and cholesterol (Fig. 6c). *In vitro* results showed that cells treated with the functionalized complex composed of EXOs and dexamethasone could modulate the immune system, reduce pro-inflammatory cytokine levels (TNF-α and IL-1β), as well as increase anti-inflammatory ones. However, the authors concluded that another encapsulation approach (*i.e.* physical nanoparticles) should be explored, as the efficiency of EXOs encapsulation did not exceed 30 % [139].

As already mentioned in this text, OA is known to be a disabling disease that impairs the tissues involved in the joint, such as cartilage, bone and ligaments. The structure and composition of the cartilage tissue undergoes changes that cause it to lose its integrity. In an effort to

restore the damaged area, chondrocytes increase their activity, which in turn generates a pro-inflammatory environment [140]. In light of such a situation, nanomaterials have been exploited as an alternative to alleviate this incapacitating syndrome, notably, as carriers of anti-inflammatory drugs [85,141]. For instance, Xue and co-workers designed a dual platform for the release of rapamycin (Rap) and bilirubin (Br) immunosuppressant and anti-ROS scavenger agent, respectively. The nanosystem was composed of different layers. In the core, rapamycin was loaded on mesoporous PDA and coated with Br-loaded metal-organic framework (MOF). To provide more site-specific delivery, the multilayer system was confined with peptide targeting type II collagen, as type II collagen is abundant in the cartilage tissue. The authors demonstrated that due to the on-demand release of Rap and Br (as PDA was sensitive to NIR light), the complex was able to prevent cell apoptosis *in vitro*. Animal studies showed the ability to target cartilage tissue and restrained cartilage degeneration by functionalizing the nanomaterial. Additionally, the latter property may be beneficial for magnetic resonance imaging *in vivo* in the future (Fig. 7) [141].

Overall, technological advances, on the one hand, have made it possible to closely replicate dynamic cartilage tissue, for instance by using bioprinters. On the other hand, nanotechnological developments have provided the means to design systems capable of influencing chondrogenesis by controlled delivery of immunomodulatory agents, either to promote regeneration or to reduce inflammation in diseases such as rheumatoid arthritis.

5. Conclusion and future outlook

BTE and CTE have taken advantage of nanotechnology to bridge the gap between the immune system and tissue engineering. Recent advances in nanostructured materials have allowed the construction of intricate systems that resemble native tissue architecture. Additionally, it has permitted controlled drug delivery of myriad of molecules (growth factors, drugs, ions). Particularly, given the role of the immune system in tissue regeneration, emerging nanomaterials have opened up new windows to delivery immunomodulatory agents that have provided enhanced systems with advantages such as more controlled release of factors or selective modulation of signaling involved in regeneration.

Although the application of these systems may represent a paradigm shift in therapeutics for treating other orthopedic-related diseases, the translation to clinics is still hampered by many hurdles. Firstly, exploiting metal-based nanomaterials such as gold or silver nanoparticles, can lead to accumulation in the organism and long-term toxicity. In the search to avoid that, an alternative approach for the translation would be to consider replacing and reducing inorganic resources with green remedies, such as algae-based nanoparticles. Secondly, bio-based immunomodulatory agents such as EVs might result in unstable biological activity, hindering to obtain consistent results and to draw clear mechanisms of action and conclusions. Hence, it is not possible to create standardized protocols and, as a result, entails regulatory issues.

Since the wide variety of both nanomaterials and cargoes may hinder the drawing of a decisive conclusion, the current issue lies in conducting more in-depth studies of each delivery platform to ensure biosafety and efficacy for future use in tissue engineering. Thus, computer science such as artificial intelligence or deep learning, yet in early stages, could be among the alternatives to provide the translation of engineered constructs to clinics, as they could analyze databases and create models to study precise mechanisms of the targeted tissue and designed system.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

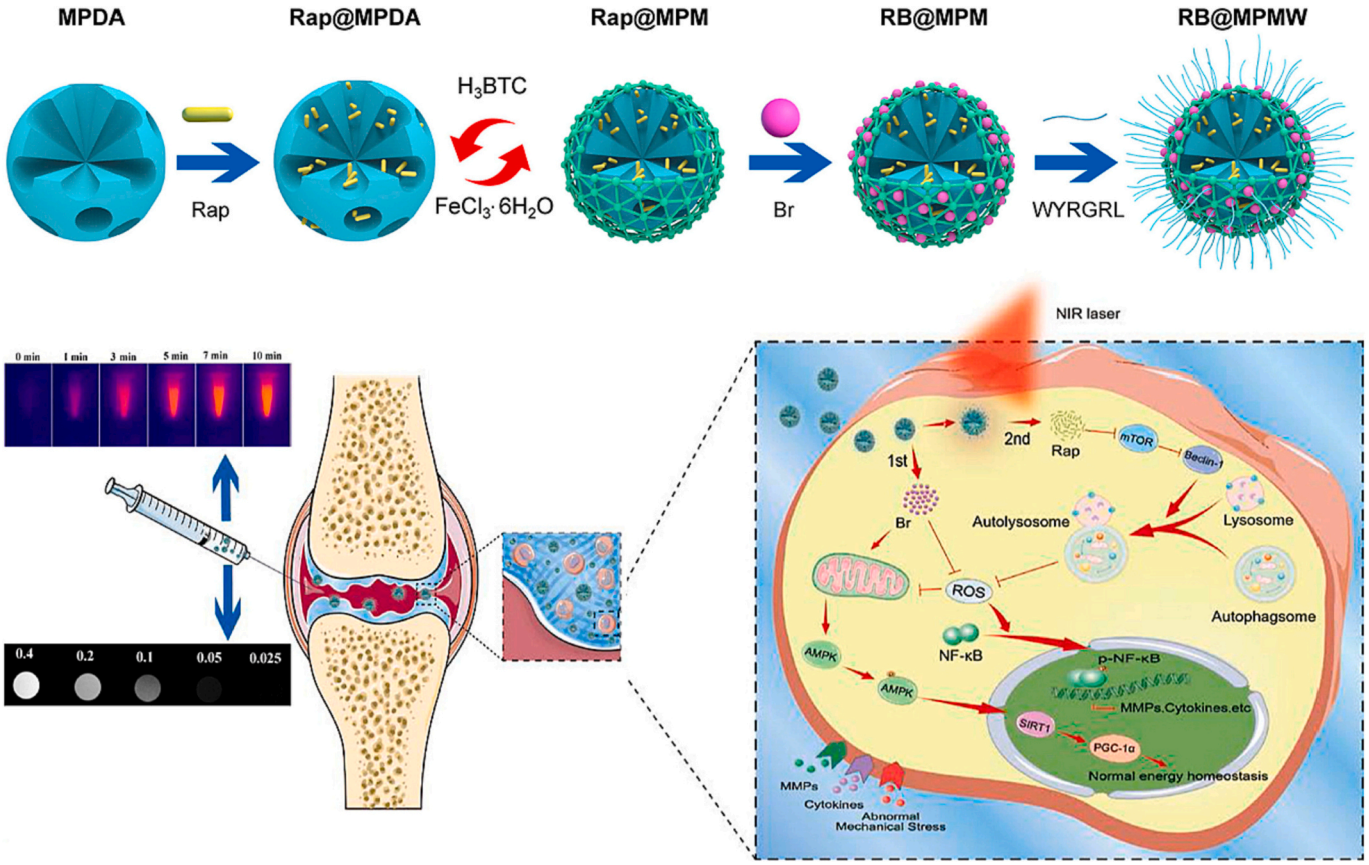

Fig. 7. Design and mechanism of action of a nanomaterial-based cartilage-targeted dual drug delivery system. Reprinted with permission from [141].

the work reported in this paper.

Data availability

No data was used for the research described in the article.

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