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Advances on gradient scaffolds for osteochondral tissue engineering

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Abstract

The osteochondral (OC) tissue is one of the most hierarchical and complex structures known and it is composed by two main compartments of hyaline articular cartilage and subchondral bone. It exhibits unique cellular and molecular transitions from the cartilage to the bone layers. OC diseases such as osteoarthritis and traumatic lesions may affect the articular cartilage, calcified cartilage (interface region) and subchondral bone, thus posing great regenerative challenges. Tissue engineering (TE) principles can offer novel technologies and combinatorial approaches that can better recapitulate the biological OC challenges and complexity in terms of biochemical, mechanical, structural and metabolic gradients, and ultimately can provide biofunctional 3D scaffolds with high reproducibility, versatility and adaptability to each patient's needs, as it occurs in OC tissue defects. The recent reports and future directions dealing with gradient scaffolds for OCTE strategies are overviewed herein. A special focus on clinical translation/regulatory approval is given.

1. Introduction

The native osteochondral (OC) tissue is considered a complex system composed by a top articular cartilage layer and an underlying subchondral bone layer interconnected by an interface region of calcified cartilage. The OC tissue is distributed in a proportion of 90% of articular cartilage, 5% of calcified cartilage and 5% of subchondral bone [1]. OC defects can arise from traumatic lesions or more commonly from natural causes. Osteoarthritis (OA) has been recognized by the World Health Organization as a public health problem affecting 240 million people globally, about 10% of men and 18.0% of women over 60 years. The main symptoms include limitations in mobility and in extreme situations people loses their ability to perform the major daily activities [2, 3]. OC defects have a limited self-healing capacity and articular cartilage is the main responsible as it is an avascular tissue. Moreover, the zonal distribution of cells, extracellular matrix (ECM) composition, mechanical properties and anisotropic structure of articular cartilage makes it even more difficult heal and mimic when replicated in OC tissue engineering (TE) approaches [4]. Deeper OC defects are characterized by affecting the calcified cartilage (interface region) and subchondral bone region. The singularity of the interfacial tissue that exhibits intermediate biological, structural, and mechanical properties of articular cartilage and subchondral bone, brings additional concerns to the medical community and tissue engineers [5–7]. Clinical findings suggest that the currently available medication can only promote palliative care to patients and do not ensure the complete healing of OC tissue defects that in most cases require surgical replacement of the tissue. However, the currently available implants (metallic and synthetic) have shown limited repair capacity, due to the lack of biological adaptive properties [8]. As alternative, autografts and allografts have been used for severe degeneration, but their insufficient supply and potential risk of viral transmission also limit their use as surgical options [9]. Thus, TE strategies show a great promise when

envisioning to overcome the limitations associated with the current treatments applied in OC regeneration. Different OCTE scaffolds have been proposed, including developed monolayered structured with the same properties on both cartilage and subchondral bone phases [10], bilayered or biphasic scaffolds detaining two different and yet connected phases [7, 11, 12], and more recently multilayered scaffolds designed with an intermediate interface-like region that connects both the articular cartilage and underlying subchondral bone-like layers [6]. An important concern for OC tissue engineers' involves the design of scaffolds with continuous gradients in terms of material composition, structure, mechanical properties, and biochemical/biological features, as these can better recapitulate the structural and biological challenges of OC tissue [13, 14]. The development of structural, mechanical and compositional gradients in OC scaffolds is possible by using classic TE strategies and making use of processing techniques such as freeze-drying [15] and salt-leaching [7, 11, 12]. These methods enable to obtain integrated gradients in terms of porosity and pore size, where the delamination of the layers is less prone to occur giving a superior mechanical support and stability to the tissue. Recently, advanced manufacturing and in particular, 3D printing methods have been shown promising results in OC tissue scaffolds fabrication [16, 17]. A major advantage of these techniques is related to the possibility of obtaining structures with well controlled pore geometries based on preconceived computational designs. Moreover, the controlled incorporation of micro and nanoparticles [18], inorganic compounds [7, 19], and nanofibers [20] also enables gradients formation for OC tissue mimicking. Thus, the scaffolds possessing hierarchical structure may be designed to exhibit matching mechanical and structural properties to OC tissue. Despite, these interesting developments, besides the need to control the segmental vascularization of the OC tissue other research avenues involving the use of anti-oxidant and anti-free radicals, and anti-microbial scaffolding approaches are also worthy of exploration in the future.

Regardless of the TE approach used for OC scaffolds development, the combination of biomaterials with cells and other biomolecules (e.g. growth factors, cytokines, hormones, nucleic acids) is extremely important for a more effective repair, replacement and regeneration of the damaged tissues [21, 22]. This strategy aims to mimic the biochemical, biophysical and biomechanical environment achieved with the ECM formation *in vitro*. The use of bioreactors as a tool to investigate cellular response but in dynamic culture conditions provides additional and more realistic information about scaffolds performance *in vivo* [15]. More recently, the use of computational modeling and reverse engineering have shown promising results in biomedical engineering research [23]. In the specific case of OCTE, data from computational modeling and laboratory experiments were combined to understand the biological processes involved in controlling osteochondral differentiation for tissue formation [24]. The physical stimuli and cellular responses for bone and cartilage formation have also been investigated [25].

Although the recent developments on OCTE have provided good outcomes for tissue regeneration *in vitro* and *in vivo*, there are still many gaps to fill and a deeper understanding of the OC gradient scaffolds long-term effects on tissue repair and regeneration is necessary.

In this review, the gradient characteristics of OC tissue are explored in terms of structural properties, cellular distribution, biochemical and metabolic composition, and mechanical behavior. Advances in OCTE strategies are considered, including recent developments in OC gradient scaffolds, processing technologies and combinatorial approaches, cell-based strategies, and mechanical stimuli under different cell culture conditions (e.g. bioreactors). We highlight the reports involving the application of computational modeling in scaffolds structure design and to predict together with experimental results the OC regeneration process. Finally, the recommendations for future directions in OC defects repair and regulatory aspects are also overviewed.

2. Osteochondral tissue complexity

OC tissue is composed of two different tissues, the articular cartilage and the subchondral bone, that transit from one layer to another by exhibiting gradient characteristics (figure 1).

The articular cartilage can be divided into non-calcified cartilage and calcified cartilage, separated by a basophilic line 'tidemark' that provides a gradual shift between the unmineralized and mineralized layers. The non-calcified cartilage consists of three zones: (a) the superficial zone, STZ (10%–20%): located in the top of the articular cartilage and interfacing the synovial fluid and joint surface); (b) the middle zone, MZ (40%–60%): located in the central region of the articular cartilage); and (c) the deep zone, DZ (30%–40%): located in the bottom of the articular cartilage) [26]. Calcified cartilage is also known as 'interface' and is located in the transition region between the cartilage and bony layers [27]. Below this region there is the subchondral bone, composed of a dense subchondral bone plate and the underlying subchondral trabecular bone [28]. The subchondral bone plate is located immediately below the calcified cartilage, followed by the trabecular bone as the deepest zone of OC tissue.

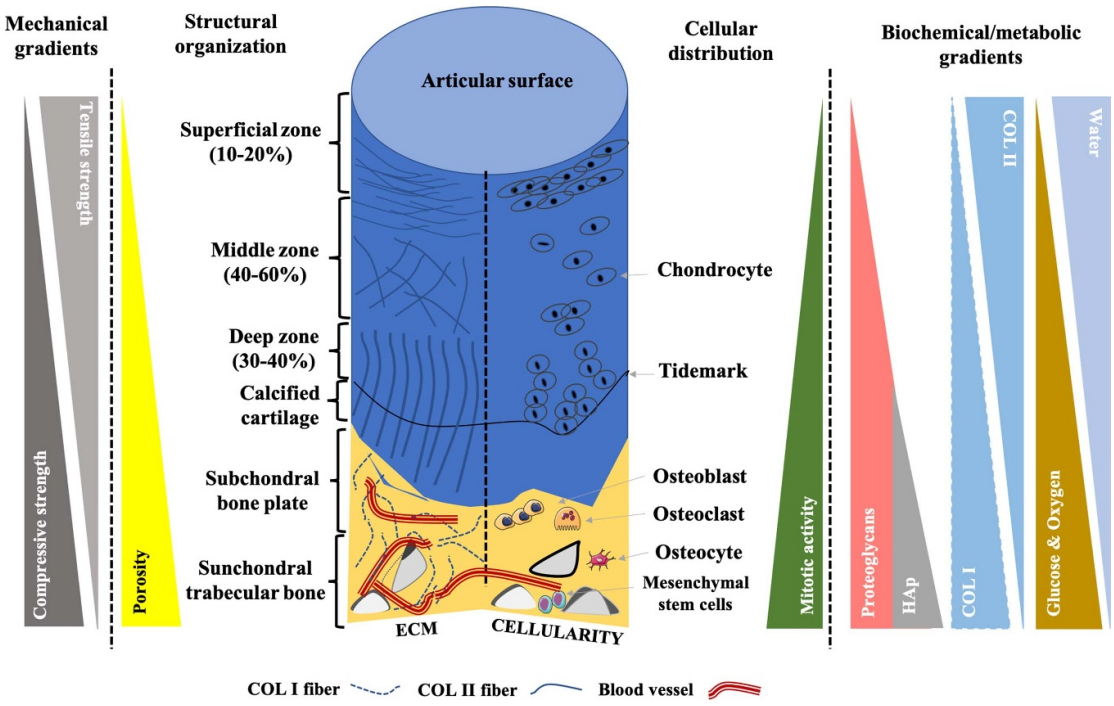

Figure 1. Schematic illustration of the zonal distribution and hierarchical properties of OC tissue. From the superficial zone (STZ) of the articular cartilage to the subchondral bone the increasing trend of compressive strength, porosity, mitotic activity, proteoglycans content, HAp amount, collagen type I content and glucose and oxygen are represented. On the opposite direction, the decreasing trend of tensile properties, collagen type II content and water are also illustrated.

The natural OC gradients defined by changes in the structural properties, cellular density and distribution, biochemical and metabolic composition, and mechanical behavior of the non-calcified cartilage, calcified cartilage and subchondral bone, are summarized in table 1.

2.1. Structural organization

The structural features of OC tissue, such as, porosity, pore size and interconnectivity are different from the surface of the articular cartilage to the bottom of subchondral bone, and are characterized by a specific anisotropic zonal distribution influenced by the ECM components, collagen fibers orientation and degree of mineralization [44]. The non-calcified articular cartilage has strong durable rubber-like nature, being 60%–85% porous and presenting reduced pore size (ranging from 2 to 6 nm). The hydraulic permeability of the tissue is small (10^{-6}–10^{-5} $m^4\,N^{-1}\,s^{-1}$), and decreases from the STZ to the DZ ensuring resistance to high mechanical forces [29]. Calcified cartilage has intermediate porosity and permeability to the non-calcified cartilage and subchondral bone. In addition, it has large pores that allow nutrients flow, cytokines and other signaling molecules to reach the upper non-calcified cartilage [27]. In the subchondral bone plate, a highly organized porous matrix can be observed presenting low porosity (5%–10%) and pore diameter (10–50 μm) [45]. Moreover, the bone plate also contains channels that link the articular cartilage to the trabecular bone (100–190 μm in humans), in order to facilitate the integration and nutrients diffusion from bone to cartilage. As for the subchondral trabecular bone, it presents an anisotropic architecture and poorly organized porous matrix, with high porosity (75%–85%), pore diameter (300–600 μm), and a decreasing volume fraction that comes from 85%–90% in the cortical bone to 5%–60% in the trabecular bone [45, 46]. The permeability of subchondral bone region is higher as compared to the articular cartilage and depends on the osteocytes density in the different bony layers [47]. The porous structures of subchondral bone are filled with blood vessels that provide nutrition and oxygen supply to trigger bone remodeling mechanisms [48].

2.2. Cellular distribution

Being a complex system, OC tissue organization also involves the distribution and density of different types of cells responsible for triggering the biological processes of articular cartilage and subchondral bone. Chondrocytes are present in the cartilage and it can represent about 2% of the components of the tissue [44]. The inability of cartilage tissue to regenerate is related to the very low mitotic activity of this cells, whose density and distribution varies along the different articular cartilage zones [30] (figure 2).

Table 1. Gradient structural, cellular, biochemical/metabolic and mechanical properties of OC native tissue.

	Structure	Cells	Biochemistry and metabolism	Mechanics	References
Non-calcified cartilage	Strong durable rubber-like nature; High porosity and low pore size; Anisotropic orientation.	Chondrocytes; Flattened morphology in the STZ to round morphology and ellipsoid shape in the DZ; Decreasing number of cells from the STZ to DZ.	Composed by proteoglycans, collagen type II and water; Decreasing water content from the STZ to the DZ; Parallel collagen fibers to the joint surface in the STZ and perpendicular in the DZ; Low oxygen levels.	Increasing compressive modulus, compressive strength and elastic modulus from the STZ to the DZ.	[26, 29–35]
Calcified cartilage	Located in the transition zone between non-calcified cartilage and subchondral bone; Intermediate porosity to the non-calcified cartilage and subchondral bone and large pore size.	Hypertrophic chondrocytes; Round morphology and giant cells.	Transition zone from the collagen fibrils that come from the DZ and anchor in the subchondral bone; Presence of collagen type X and alkaline phosphatase.	Mechanical transition between the flexible cartilage and the rigid subchondral bone; High elastic modulus due to the presence of a mineralized matrix.	[27, 36–39]
Subchondral bone	Dense subchondral bone plate with high low porosity and pore size; Isotropic trabecular bone region with high porosity and pore size.	Osteoblasts, osteoclasts, osteocytes and mesenchymal stem cells; Osteocytes as the most abundant type of cells.	Composed of an organic phase, a mineral phase, and water; Collagen type I, glycoproteins and proteoglycans constitute the organic phase; Hydroxyapatite (HAp) is the main component of the inorganic phase; High oxygen levels.	Ensures the integrity of the articular cartilage and osteochondral tissue; Anisotropic mechanical behavior with different tensile and compressive properties in the longitudinal and transverse direction.	[35, 40–43]

STZ: superficial zone; MZ: middle zone; DZ: deep zone.

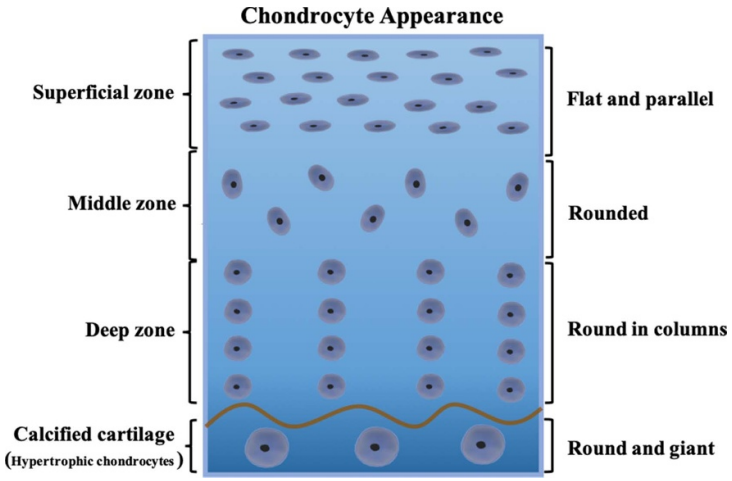

Figure 2. Schematic illustration of chondrocytes density and distribution along the different articular cartilage zones.

In the STZ, the highest number of cells ($\sim 24 \times 10^3$ cells mm^{-3}) can be found exhibiting a flattened morphology and being aligned parallel to the joint surface. Herein, cells are able of secreting a specialized type of proteins that facilitate the lubrication of the tissue. The MD zone, contains chondrocytes with more rounded shape and in a much lower number as compared to the STZ zone ($\sim 10 \times 10^3$ cells mm^{-3}). The fewest number of chondrocytes ($\sim 8 \times 10^3$ cells mm^{-3}) that compose the DZ also exhibit round morphology and are aligned in columns [31, 32]. The calcified cartilage zone is considered as a transition part between the more flexible cartilage and the rigid subchondral bone, herein the limited amount of chondrocytes present hypertrophic phenotype being 20 times larger than the normal chondrocytes [36, 37]. In the bottom region of OC tissue, the subchondral bone composed by a dense subchondral bone plate and a subchondral trabecular bone region contains four different cell types (osteoblasts, osteoclasts, osteocytes and mesenchymal stem cells (MSCs)) responsible for ECM formation and bone regeneration. Osteoblasts are proliferative cells with high mitotic activity and responsible for bone formation, but represent only 4%–6% of the total cell number in bone tissue. The most abundant cells are the osteocytes (90%–95% of the total cell number), originated as mature osteoblasts and regulators of osteoblasts and osteoclasts activity. In respect to cell density, it is believed that the trabecular bone presents a superior cellular component as compared to that for the dense subchondral bone plate [41].

2.3. Biochemical and metabolic gradients

The two main compartments of OC tissue, i.e. hyaline articular cartilage and the subchondral bone, also exhibits a transition of biochemical and metabolic gradients from one compartment to another. Articular cartilage is mainly composed by proteoglycans (e.g. aggrecan, decorin and fibromodulin), collagen type II and water, varying their distribution and concentration according to the cartilage zone [33]. Water and electrolytes compose about 60%–80% of the total weight of the articular cartilage, decreasing from 80% to 65% from the STZ to the DZ. The remaining 20%–40%, are ECM components responsible for stabilizing the collagen type II fiber network. Attached to the proteoglycans are found the glycosaminoglycans (e.g. chondroitin and keratin sulfates) that have a high density of negative charges attracting osmotically active cations and absorbing water [31, 49]. Being an avascular tissue with hypoxic bioenvironment, articular cartilage can obtain their nutrients and oxygen from the synovial fluid that surrounds the cartilage surface [34]. Thus, chondrocytes are supplied with low amounts of oxygen. These cells are considered as glycolytic ensuring the constant supply of glucose for helping on proteoglycans synthesis and GAG's regulation along the different cartilage zones [50]. In the STZ, proteoglycans constitute 15% of articular cartilage dry weight and the thin collagen fibers (30–35 nm in diameter) represent 86% of articular cartilage dry weight, being densely packed and parallel to the cartilage surface. In the MD zone, proteoglycans are abundant (25% of articular cartilage dry weight) and collagen fibers' diameter increases as its amount decreases (67% of articular cartilage dry weight), being obliquely orientated to the cartilage surface. The remaining thickness of the tissue is taken up by the DZ, where proteoglycans are still present in high amounts (20% of articular cartilage dry weight) but the amount of collagen fibers reduces in this region while the size of fibers is the highest from all articular cartilage (40–80 nm in diameter), being perpendicularly orientated to the articular surface and anchoring in the underlying subchondral bone [31, 32, 44]. Thus, the calcified cartilage zone receives the collagen fibrils from the DZ through this area until reaching the subchondral bone. Collagen

type X, as the main product of hypertrophic chondrocytes, and alkaline phosphatase are present in this region, thus indicating similarities to the underlying subchondral bone tissue [38]. Since the blood vessels are the oxygen and nutrients source of subchondral bone, both DZ and calcified cartilage will also be provided by metabolites that come from this layer. The dense bone plate and trabecular bone of the subchondral layer are composed of an organic phase, a mineral phase, and water. In the organic phase, it can be found the bone tissue cells surrounded by an ECM of collagen type I and other non-collagenous proteins (e.g. glycoproteins and proteoglycans). The inorganic phase is mainly formed by HAp deposited within the organic phase to form bone tissue [41]. In general, HAp constitutes 60% of bone tissue dry weight, but in OC tissue it is present in gradients: ∼85.8% in the subchondral bone, ∼65.1% in the calcified cartilage, and 0% in the chondral layer. The remaining 10% of the tissue is filled with water, penetrating the porous structures and interacting with the collagen fibrils and the mineral crystals [37].

2.4. Mechanical gradients

The mechanical properties of OC tissue vary from the surface of the articular cartilage to the subchondral bone as a result of the heterogeneous composition and anisotropic structure of the tissue that distributes the mechanical stresses along the different regions and interfaces. The compressive modulus of cartilage shows depth-dependent variations due to changes in the cellular, biochemical and structural composition from the STZ to the DZ zone. The proteoglycans content, collagen fibers orientation and diameter, as well as, the viscoelastic nature of the tissue, or the cell density and distribution along the different zones are responsible for this variation [35]. Thus, from a functional point of view articular cartilage is divided in two principal zones. The STZ ensures the normal mechanical function of cartilage tissue and is responsible for the tensile strength and compressive loads distribution. The MD and DZ are together responsible for supporting the compressive loads of the tissue, which allows it to recover from the impacts felt in the STZ [51]. Thus, the compressive modulus and compressive strength of articular cartilage increase from the top to the bottom, with values ranging from 0.2–6.44 MPa to 0.005–4 MPa, respectively [26]. A gradient of elastic modulus values ranging from 0.02 MPa in the STZ to 6.44 MPa in the calcified cartilage has also been measured [35]. The calcified cartilage zone is considered essential for the mechanical transition between the flexible cartilage and the rigid subchondral bone, minimizing the stiffness gradient between the two tissues [39]. The elastic modulus in the calcified cartilage can reach values of ∼0.3 GPa due to the presence of minerals, absent in the remaining cartilage zones [37]. Finally, the integrity of the whole articular cartilage and OC tissue is ensured by the subchondral bone, namely by the bone tissue properties: i.e. porosity, collagen fibers orientation and higher degree of mineralization [52]. The arrangement of the organic and inorganic components provides bone tissue anisotropic properties, which in turn leads to different mechanical behavior. For instance, the tensile and compressive strength of compact bone plate in longitudinal direction are ∼135 MPa and ∼205 MPa, respectively. In the transverse direction these values change to ∼53 MPa and ∼131 MPa, respectively. As for the compressive modulus, in the transverse direction the values reach ∼10 GPa, whereas in the longitudinal direction these are of ∼18 GPa [42]. Trabecular bone shows superior mechanical properties under compression than tension, with a compressive modulus of 1–900 MPa and a compressive strength of 1–10 MPa [35, 43].

3. Advances in OCTE

The complexity of OC tissue related to the specific physiological, mechanical and structural properties of its composing tissues (i.e. cartilage, interface and subchondral bone), enables to classify OC defects into five different grades: (a) Grade 0, normal and healthy articular cartilage; (b) Grade I, swelling and softening of articular cartilage surface; (c) Grade II, partial thickness articular cartilage defects; (d) Grade III, defects cover the entire articular cartilage and interface; (e) Grade IV, defects reach the subchondral bone [53, 54] (figure 3).

Thus, the design of novel strategies capable to respond to the different grades of OC defects has been one of the main focus of researchers in TE and regenerative medicine (TERM). Current treatments of OC defects can be divided into non-surgical and surgical according to the grade of the defect. Non-surgical treatments include the use of medication (e.g. commercial chondroitin sulfate, hyaluronic acid, glucosamine) and physiotherapy that can help to relive the pain and improve the function of tissues with small grade defects (Grade II). Nevertheless, for defect regions of critical size (Grade III and IV) the non-surgical treatments fail, giving way to surgical options that can be more effective for OC tissue repair/restore, i.e. arthroplasty, chondroplasty, microfracture, ACI, bone marrow aspirate concentrate, implantation of natural/artificial bone, osteochondral autografts and allografts transplantation [55]. Although these surgical options have been widely applied in over than 2 million surgeries per year [56], long-term failure caused by slow

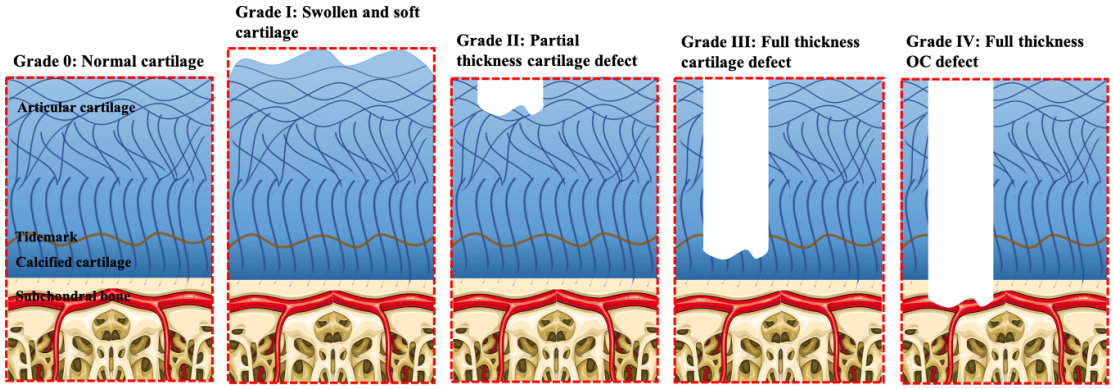

Figure 3. Schematic illustration of the different types (grades) of OC defects.

remodeling, immune reactions and secondary traumatization, demand for advanced therapies capable of better respond to patients needs and provide the complete healing and regeneration of OC defects.

3.1. Types of biomaterials

New TERM strategies are appealing to overcome the limitations of current surgical treatments of large OC defects, as they might provide a faster regenerative process and fully integrated neo-tissue, avoiding secondary reactions and failure of treatments. The type of TERM strategies will be dependent on the OC defect category, stage, size and locations. Moreover, the combinatory effects of biomaterials, cells and biological factors are the key for effective TERM approaches [54].

Biomaterials are considered the backbone of 3D engineered scaffolds, supporting tissue growth, mechanical strength and structural stability for an accurate regenerative process. Moreover, biomaterials are responsible for providing the biomimetic microenvironment recognized by cells to proliferate and produce specific ECM. Biomaterial scaffolds can be derived from natural polymers, synthetic polymers, metallic materials, bioceramics and decellularized ECM-based materials [1, 57]. Naturally derived polymers have been extensively explored for bone [58], cartilage [59], and OC [7, 11, 12] TERM due to their biocompatibility and biodegradability. They can be classified into polysaccharides (e.g. glycosaminoglycans, cellulose or chitin) and proteins (e.g. collagen, gelatin and silk fibroin), constituting tissues ECM and benefit microenvironment for cellular response. The binding motifs naturally present in biopolymers makes them an eligible choice for TE strategies, supporting cell attachment, viability, proliferation, and differentiation, in most cases at higher extent compared to synthetic polymers. Nevertheless, their rapid degradation, limited processability and poor mechanical properties are considered important drawbacks that can limit the processing of natural-based scaffolds [60]. Previous studies, showed that chemical backbone modifications of naturally derived polymeric materials [61, 62], or their blending with synthetic polymers [63, 64], are possible solutions for improving scaffolds performance in OCTE strategies. Furthermore, the mechanical and morphological properties (e.g. porosity) of OC tissue constructs can also be tuned by adjusting the crosslinking reactions of biopolymers, and selecting cell binding motifs (e.g. arginine-glycine-aspartic acid sequence) and matrix metalloproteinases capable of maintaining the bioactivity of materials and improving their structural performance [54]. On contrary to the limitations presented by natural-based polymers, synthetic polymers have shown potential for OCTE applications due to their improved mechanical properties and slow degradation rates. Moreover, they are easily chemically modified and engineered with specific structural properties [65]. Among the possibilities, the resorbable synthetic polymers poly lactic-*co*-glycolic acid (PLGA), poly(ethylene glycol), and polylactic acid (PLA) are the principal synthetic matrices used in OC and cartilage TE strategies [66]. However, the lack of biologically active domains pursued for their blending with naturally derived polymers [64], and functionalization with cell binding cues [67]. It is well known that bioinert ceramics, bioactive ceramics (e.g. bioglasses) and bioresorbable ceramics (e.g. HAp and calcium phosphates) are of particular interest for bone regeneration purposes, presenting excellent osteoconductive properties and bioresorbability [1]. Over the past years, the blending or incorporation of bioceramics into polymeric matrices have been proposed to complete the subchondral bone regeneration process, taking advantage of ceramic compounds for bonding osteogenesis and the polymeric matrices to improve the elastic modulus [68]. More recently, bioactive ions, such as, lithium, strontium, zinc and manganese, have shown to be essential for OC tissue regeneration when doped into bioresorbable ceramics like β-tricalcium phosphate [7, 11, 12]. Metallic implants remains the standard choice for more extreme situations, in which the entire

articular cartilage or OC tissue needs to be replaced [69]. Usually, metallic implants used as bone substitutes (e.g. titanium and cobalt-chromium) have a common feature of biological inertness. However, their potential bioactivity and biodegradability has been explored in recent years. Bioresorbable ceramic particles have been successfully proposed for coating metallic implants and improve apatite-like crystal formation for implants fixation into the subchondral bone [70]. Finally, the dECM-based biomaterials derived from tissues and cell-culture systems have recently gained attention for their proximity to the *in vivo* scenario, as they reunite the entire components of tissues ECM to be repaired or mimicked. Thus, dECM's are ideal for controlling cell behavior and tissues homeostasis without inducing antagonist immune responses [71]. The possibility of replicate tissues' microenvironment through these matrices, inspired researchers to use dECM's from articular cartilage and demineralized bone matrix for OC tissue regeneration [72, 73]. Nevertheless, the decellularization process and ECM preservation typically combine exhaustive protocols of mechanical, physical and chemical manipulation that diminish the mechanical properties of the dECM-based scaffolds. Thus, it has been common the development of biohybrid materials composed of dECM's and synthetic polymers that ensure superior mechanical performance [72]. Ultimately, each type of biomaterial has its own advantages and disadvantages, but it is always necessary to find a balance between them in order to choose the best biomaterial composition for a specific OCTE application.

3.2. Processing methods of scaffolds for osteochondral regeneration

Over the last decades, several TE processing methods have been proposed for 3D scaffolds development and application in OC regeneration research. Simple strategies designed for individually regenerate bone or cartilage tissues are based on methodologies such as electrospinning, phase separation, gas-foaming, freeze-drying, solvent-casting and sol-gel methods [1]. Moreover, the combination of such methodologies revealed to be effective in the creation of more complex structures that can simultaneously simulate the hierarchical and gradient microstructure of articular cartilage and subchondral bone in OC tissue [8]. Electrospinning has proved to be an effective and versatile technology for creating ECM-mimicking scaffolds, presenting randomly organized fiber networks that improve cell adhesion, proliferation and ECM formation. However, the limited mechanical properties and low three-dimensionality of electrospinning structures are relevant issues for OCTE purposes. Phase separation and solvent-casting methods have been successfully proposed to produce 3D porous OC scaffolds with high mechanical properties, macro-/micro-porosity and interconnectivity, mimicking subchondral bone architecture [7, 11, 12, 59]. Gas-foaming method has the advantage of preparing scaffolds with controllable shape and density, but the formed pores are disconnected and usually form a low porosity. Moreover, the mechanical properties obtained are not comparable to those of phase separation and solvent-casting methods, which hinders their application for OCTE purposes. As for freeze-drying, this method is extensively used for scaffolds preparation and storage, and recently it has been shown that it can be successfully applied for the development of hierarchical structures that mimic OC tissue [15]. Sol-gel method is applied for hydrogels formation involving gently reaction temperatures suitable for cell-encapsulation strategies [74]. The reported scaffolding method is suitable for articular cartilage TE owing to high hydration degree and low porosity that mimic tissue ECM and architecture [75]. Recently, 3D printing technologies emerged as convenient and promising methods for developing OC scaffolds with controlled architecture and pre-defined porosity. This method stands from the others as researchers can through a single processing mimic the anisotropic and heterogenous properties of articular cartilage and subchondral bone, recreating the different OC layers only by adjusting the printing parameters [76]. Moreover, 3D printed scaffolds can be designed according to specific OC defects envisioning patient-specific approaches [77]. Scaffold-based 3D printing can be categorized into cellular and acellular approaches, meaning 3D printing and 3D bioprinting, respectively. The 3D bioprinted scaffolds (cellularized) can be developed by different methods such as: (a) extrusion-based printing, (b) laser-based printing, (c) droplet-based printing and (d) stereolithography. The 3D printed scaffolds (acellular) are usually created using fused deposition modelling, melt electrospinning writing and selective laser sintering [1]. Both strategies have been used to prepare multilayered scaffolds with articular cartilage, interface and subchondral bone-like layers, demonstrating adequate cell behavior from both seeded and encapsulated cells for osteochondral regeneration [76, 78]. *In vivo* studies, showed that 3D printed OC scaffolds have potential for OC defects regeneration on both articular cartilage and subchondral bone defect regions [79]. Over the last years, biomaterials at nanoscale have been playing a central role in TE [53]. Nanoparticles can be included/incorporated into 3D scaffold materials and used to target cells and better control drug release, genes and growth factors delivery for OC regeneration. Thus, they are usually applied as an additional strategy to complement biomaterials properties of mechanical and functional support. Previous studies, proposed different natural and synthetic-based biomaterials, including collagen, silk fibroin, PLA and PLGA, to be used as supporting matrices of nanoparticles aiming to stimulate articular cartilage repair and promote subchondral bone formation [80–83]. Among the possibilities, lipids, dendrimers, silica and gold

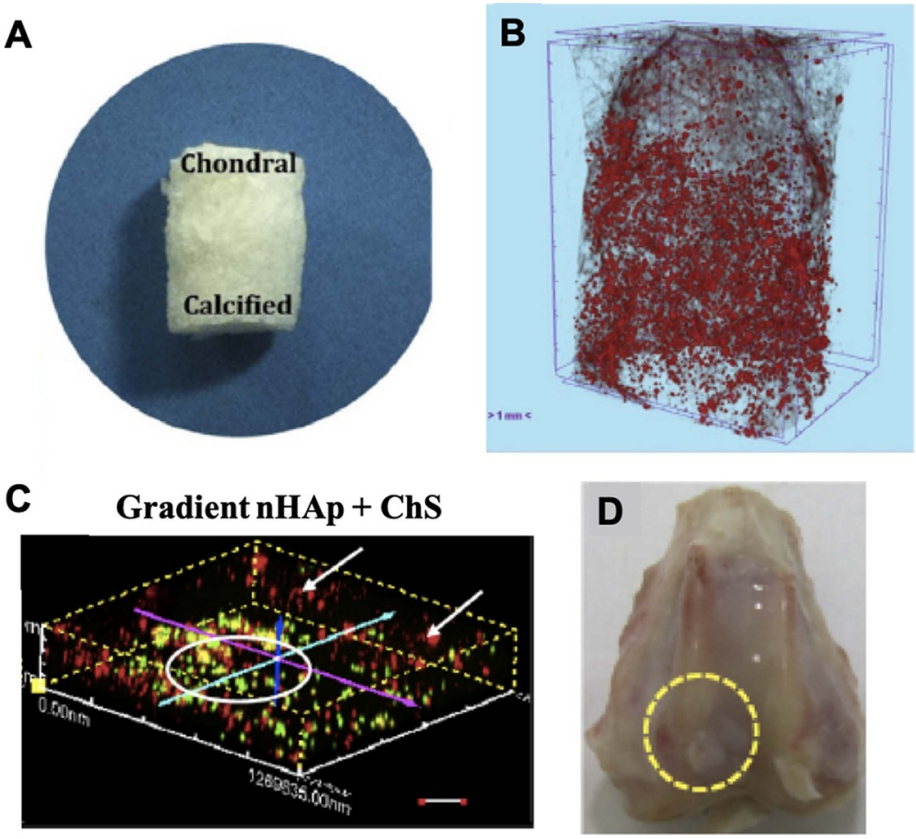

Figure 4. Nanoengineered OC gradient hydrogels. (A) Optical image of lyophilized hydrogel. (B) Micro-CT 3D analysis of gradient hydrogel showing the nHA particles (red) alone distributed in gradient pattern. (C) Co-culture of primary rat osteoblasts (red) and caprine chondrocytes (green) in the gradient hydrogel after 3 d of culture (scale bar: 250 μm). (D) Gross appearance of defect site after 8 weeks of surgery in the nHA/ChS gradient hydrogels. Reprinted from [84], Copyright (2018), with permission from Elsevier.

nanoparticles have been proposed for OCTE applications [18]. Previously, an alginate/PVA blend hydrogel network containing chondroitin sulfate nanoparticles (ChS-NPs) in the chondral region and nanohydroxyapatite in the subchondral zone, was proposed for OC regeneration [84]. The work showed that co-cultured osteoclasts and chondrocytes in the nanoengineered gradient scaffolds were successfully retained in the respective chondral and subchondral regions and presented cell–cell interactions at the interface. Moreover, the gradient hydrogels showed complete closure of OC defects after 8 weeks of implantation using a rabbit model (figure 4).

Nanoparticles loaded with bone morphogenetic protein-2 (BMP-2), BMP-7 and transforming growth factor-β (TGF-β) have shown potential to improve OC regeneration, i.e. it supported articular cartilage regeneration and subchondral bone formation [85, 86]. Due to the long-term expression efficiency, the controlled delivery of therapeutic genes become a promising strategy in the use of nanobiomaterials for articular cartilage [87, 88] and subchondral bone [89, 90] regeneration. As an example, bilayered scaffolds composed of type I collagen in the articular cartilage-like layer and HAp nanocrystals in the subchondral bone-like layer were incorporated with multi-shell nanoparticles (i.e. a calcium phosphate core and DNA/calcium phosphate shells) conjugated with polyethyleneimine to delivery pDNA encoding BMP-2 and TGF-β3, respectively [22]. Results showed that the sustained release of pDNA from matrices induced prolonged effects on MSCs osteogenic and chondrogenic differentiation, which may enhance scaffolds performance for accelerating OC regeneration *in vivo*.

3.3. Bioinspired strategies for osteochondral gradient scaffolds development

Gradients are an important feature of complex OC tissue and are imperative to recreate in scaffolds' design for the simultaneous reconstruction of chondral and subchondral defects. Previous section focused on the different TE methods that can used for OC gradient scaffolds fabrication, i.e. electrospinning [91], phase separation and solvent-casting [92], gas-foaming [93], and 3D printing [16]. Amongst the most important strategies that can be followed to form those gradients comprehending the overviewed biomaterials, additive manufacturing techniques are the most versatile. These can enable to fabricate 3D scaffolds with high control over the internal architecture (e.g. porosity, pore size and volume fraction) and external shape of the

scaffolds (macro-level). Among them, the aggregation of materials by sequential layering of solid biomaterials is a strong possibility. As example, the use of adhesives (i.e. solvent glues) or bioadhesives (e.g. fibrin-glue or alginate-boronic acid) has been proposed to bind different polymeric structures as a single OC scaffold [94, 95]. However, the interface bounding's are usually not stronger enough to preserve the required integrity of the whole scaffold, resulting in delamination between layers. Thus, other works focus their research on developing integrated bilayered and multilayered scaffolds without requiring the use intermediate adhesives [11–13]. As an example, the addition of a liquid polymeric network to molded scaffolds followed by crosslinking can be sequentially repeated to build layered structures. This approach has been previously proposed by Ribeiro *et al* [7] to create structural and compositional gradients in silk fibroin-based OC scaffolds. Researchers from the University College of London proposed a novel OC scaffold based on titanium, PLA, and a collagen-PLGA, showing that the dense junction layer of PLA used as calcified cartilage-like layer provided stability and a structural gradient (i.e. in terms of mechanical properties and composition) to the multilayered scaffolds [3]. In a different approach, Canadas and coworkers reported a freeze-drying technology using multi-steps of lyophilization and temperature gradients to create 3D OC tissue models with gradient porosity and distribution of HAp microparticles [15]. Nevertheless, an inert limitation dictates the application of these methods, i.e. the small size of the created interface layers that makes them transition layers rather than gradient layers. Thus, other methods have been proposed to achieve greater structural complexity of OC scaffolds, including 3D printing. Making use of 3D printing, the articular cartilage, interface and subchondral layers can be structurally well-distinguished and mechanical gradients can be assembled by simply adjusting printing parameters and inks composition [17]. Different studies, reported the ability to create structural and compositional gradients through 3D printing technologies and spatially modulate stem cells differentiation down chondrogenic and osteogenic lineage [19, 96–98]. These examples focus on cell seeding strategies, but as alternative 3D bioprinting can be used based on cell-laden bioinks [99]. That strategy has the advantage of directly create cellular gradients into the scaffolds with spatial control that automatically generate multiple gradients within whole matrices. Hu *et al* [99], prepared bioinks using different proportions of chitosan, gelatin and hyaluronic acid, inoculating bone MSCs. Results showed that cells distribution influenced the mechanical gradients on the matrices which in turns affect cell differentiation pattern for tissue repair.

Another approach, includes the incorporation of growth factors as gradient making systems into single polymeric structures. Wang *et al* [100] used microspheres for the incorporation of insulin-like growth factor-1 (IGF-1) and BMP-2 into single alginate and silk fibroin hydrogel scaffolds. The results showed that scaffolds exhibited deep and linear concentration gradients induced by the factors affecting human MSCs chondrogenic and osteogenic differentiation.

The aforementioned examples remote to the direct deposition of materials, particles, cells or biologically active factors for creating gradient scaffolds for OC applications. Nevertheless, alternative approaches might be used in which a starting homogeneous system is physically influenced (i.e. applied forces) to redistribute components and form gradients [101]. Another example, the buoyancy method or spontaneous de-mixing process was proposed for OC scaffolds fabrication in which the density differences between mixed fluids lead to the formation of materials with tunable structural, mechanical and compositional gradients [102]. The biochemical gradients included into cell-laden gelatin methacryloyl hydrogels, locally stimulated osteogenic differentiation and matrix mineralization during OC tissue formation (figure 5).

Other approaches may include the use of electric or magnetic fields to direct TE components distribution for gradients formation [103]. Xu *et al* [104] proposed to use an electric field to guide β-sheet silk fibroin nanofibers migration and tune the hydrogelation rate of different hydrogel systems. With this, mechanical and compositional gradients were formed capable of control osteogenesis and chondrogenesis in ectopic OC tissue regeneration.

The discussed methods involve gradients formation during scaffolds processing. Nevertheless, these are not exclusive and post modifications of pre-formed scaffolds have been explored for biochemical gradients inclusion into OC scaffolds. Dorcemus *et al* [105] showed that the gradual diffusion/adsorption of BMP-2 solution component could be used to form biochemical gradients on PLGA/hyaluronic acid scaffolds. The proposed OC structures showed spatial distribution of the growth factor throughout the scaffold along with spatial osteogenic differentiation of human MSCs and osteogenesis by marked increase of mineralized matrix.

3.4. Cellular-derived strategies and culturing conditions, and 3D *in vitro* models

Autologous chondrocytes implantation (ACI) has been clinically applied for decades. The ACI technique allows achieving satisfactory long-term results (84%–90%) in patients with different types of single femoral condyle lesions, whereas patients with other types of lesions have a lower degree of success (mean of 74%) [106].

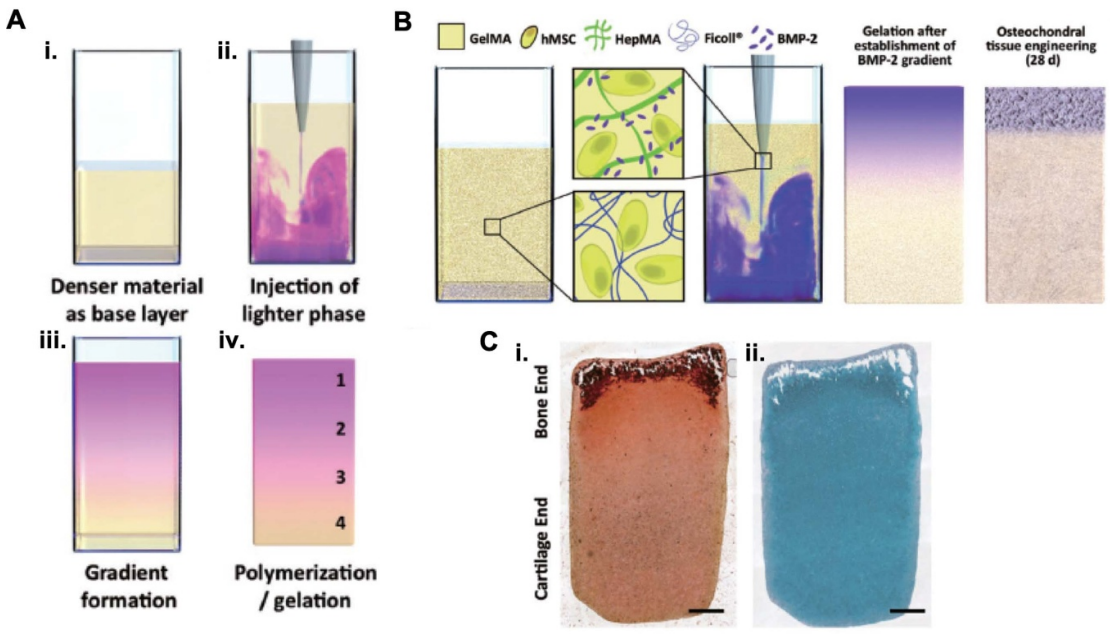

Figure 5. Buoyancy method used for developing OC gradient scaffolds. (A) Schematic showing the steps of gradient formation: (i) a base layer (yellow) is added to a mold, (ii) the injection phase (purple) is introduced during a single injection at a controlled rate, (iii) the system is allowed to equilibrate and form a material gradient, and (iv) the gradient is preserved by gelling or polymerizing the material. (B) Biochemical gradients formation through buoyancy method in human MSCs-laden gelatin methacryloyl hydrogels. (C) OC tissue formation stained with: (i) Alizarin Red S for observing mineralized matrix, and (ii) Alcian Blue to visualize sulfated glycosaminoglycans distribution (scale bar: 1 mm). Reproduced from [102]. CC BY 4.0. © 2019 The Authors. Published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Other cellular derived approaches involving the use of MSCs and platelet-rich plasma (PRP) in the treatment of OC defects have been applied mostly to small size cartilage defects and management of symptoms in patients with OA. Despite its use in the clinics is still being controversial, the therapeutic use of MSCs and PRP, alone or in combination, is traditionally related to both their anti-inflammatory activity and differentiation potential. In 2020, a clinical trial has demonstrated that the intra-articular injection of bone marrow-derived, culture-expanded MSCs with or without the addition of PRP is effective in improving function and decreasing symptoms at 12 month follow-up [107].

The advantage of considering MSC-based therapies has been attributed to paracrine secretion. In particular, the use of exosomes, i.e. the cell-secreted bi-lipid membrane and nano-sized extracellular vesicles of about 50–100 nm in size, have been attracting a great deal of interest. It has been reported by Zhang *et al* [108] that the intra-articular injection of human embryonic MSC-derived exosomes have a beneficial effect in cartilage repair. That pre-clinical study approach in a rat model has demonstrated that the use of exosomes is an appealing alternative to cell-based MSC therapy.

Cell culture conditions are important key factors for the development of effective treatment solutions for OC regeneration. Traditional static cultures can provide an initial screening of cell behavior within proposed scaffolds matrices. However, in OCTE strategies culture medium conditions need to be adjusted according to the tissue layer, i.e. by means of using different medium and growth factors for bone and cartilage, and maintained over the culture time-period in order to ensure reproducibility, standardization, and ECM proteins formation during tissue regeneration [109]. Depending on the OC tissue layer different growth factors are responsible for maintaining both bone and cartilage activity. In case of articular cartilage tissue formation, the growth factors responsible for chondrogenic differentiation include transforming growth factor (TGF-β), dexamethasone and ascorbic acid [59]. These can be applied in micromass culture models, pellet cultures alone or included in hydrogel matrices, or in cell seeded porous scaffolds [59, 110, 111]. As for subchondral bone formation, dexamethasone, β-glycerophosphate and ascorbic acids additives comprise the osteoinductive medium, which in turn enhance the function of specific growth factors of bone, including platelet-derived growth factor, fibroblast growth factor, IGF, and TGF-β [112]. Cells seeded in highly porous and interconnected structures may receive these growth factors to activate signaling pathways that guide cells to differentiate and produce ECM proteins necessary for bone regeneration [58]. These strategies may be interesting to evaluate the individual behavior of cells in the separate layers of OC-based scaffolds. However, the application of chondrogenic and osteogenic growth factors in a combined culture medium can induce

heterogeneous cell behaviors since the requirements for bone and cartilage are different [112]. Although the static loading of cells into the scaffolds making use of micropipetting technique is the most common for cell seeding in static conditions, this methods usually offers a very low seeding efficiency a non-uniform cell distribution within the structures. To overcome such limitations, bioreactors and microfluidic devices have been designed to provide a dynamic stimulus element for cells seeding and OC tissue *in vitro* culture [113]. These might be used as an alternative or combined with the previously listed growth factors for the signaling part of the TE approach. Usually, their design is based on the type of strategy, OC defect and scaffolds used. OC defects can reach many millimeters, which makes it difficult to mimic using low size scaffolds cultured under static conditions. For instance, the mineralized bone matrix can reach $\sim$240 μm in depth, and in most cases, osteoblasts cultured in subchondral bone grafts cannot reach this depth. Thus, the use of bioreactors will provide an adequate microenvironment to mimic the entire structure of OC tissue and defects. The designed bioreactors of OC grafts have been evolving, as well as, its complexity to recreate the OC tissue and/or defects. The first generation bioreactors for OCTE are the spinner-flasks which consist of a container and stirring element for the continuous stirring of the cell suspension [113]. They allow for the culture medium to reach the entire cell volume placed in contact with large size scaffolds. However, a high amount of medium is necessary due to the shear forces of the stirring bar. As an example, scaffolds of high porosity and pore size have shown an uniform cell distribution when dynamically cultured in spinner-flask bioreactors [114]. The rotating bioreactors are specially interesting as the whole container rotates instead of only the culture medium by stirring [113]. Using that devices allows to perform chondrogenic and osteogenic dynamic cultures with adjustable shear forces to influence cell behavior over time. For instance, chondrocytes seeded in a rotation bioreactor with low rotation speed showed an efficient cell adhesion, and formed large aggregates over time with the increase of rotation speed [115]. Chitosan/gelatin hybrid hydrogels encapsulated with human adipose derived-stem cells (hASCs) and cultured under dynamic condition in a rotation bioreactor, showed extensive expression of ECM, uniform cell distribution an full infiltration of cells and ECM inside the porous scaffolds [116]. A different class of compression bioreactor's systems was designed with the purpose of exert controllable mechanical stimuli under physiological conditions reproducing specific tissue properties. For OCTE strategies, the main applications of these systems are in the articular cartilage region, as it is the first contact of mechanical stresses suffered in the joints [117]. Different types of compression bioreactors have been proposed, including cyclic compression bioreactors [118], rolling compression bioreactors [119, 120], and perfused compression bioreactors [121]. Hydrodynamic shear, mechanical compression, tension and friction, or hydrostatic pressure are some of the mechanical forces promoted by these class of bioreactors simulating articular cartilage stimuli [122]. Another step within the development of bioreactors was the introduction of continuous perfusion flow in the dynamic system. The perfusion flow bioreactors provide several improvements such as stable nutrient supply, immediate waste metabolites and control of oxygen tension distribution. Importantly, the vasculature network is prone to be simulated by the shear stresses that affect cell morphology and gene expression [113]. Within these dynamic systems, the cyclic mechanical stresses modulate growth factors secretion and simulate the chondrogenesis and osteogenesis promoted by cells. Moreover, cell diffusion and spreading throughout the entire scaffolds volume is enhanced together with improved seeding efficiency. Different flow perfusion bioreactors have been proposed for OC purposes [123–125], and the possibility of simultaneously and independently culturing chondrocyte and osteoblasts as representatives of the articular cartilage and subchondral bone regions, is the hallmark of these systems. As example, Canadas *et al* [15] fabricated 3D porous structures of linear (anisotropic) and random (isotropic) porosity and containing a gradient of HAp in order to simulate OC tissue formation. A dual chamber bioreactor was used to promote a gradient of growth factors from the top to the underlying layers of the scaffolds, together with the co-differentiation of fat pad ASCs in the respective layers of the hierarchical scaffolds (figure 6).

Results showed osteogenic and chondrogenic markers expression with spatial control, as well as, a pre-vasculature observed in the bone-like layer induce by the perfusion flow of pro-angiogenic medium in this region. The interface region of OC tissue was also represented by the distinct patters of osteogenic and chondrogenic markers expressed in the intermediate region of scaffolds. In a different study [126], the co-culture of osteoblasts and chondrocyte-like cells performed in bilayered scaffolds and using a dual-chamber bioreactor revealed OC tissue growth induced by the different culture media perfusing the chambers. The proposed system provided the desired microenvironment to simultaneously grow an articular cartilage and a subchondral bone region simulating OC tissue and defects.

Microfluidic devices allow to spatially control at micrometer scale and with high resolutions and sensitivity the dynamic fluids that contacts with cells, molecules and matrices, allowing their exploitation to engineer complex tissues as 3D *in vitro* models. In the particular case of OC tissue, the outperformance of microfluidic models is due to the possibility of forming structural, biochemical, and even mechanical gradients as the most relevant properties of the tissue [127]. The 3D OC implants can be integrated with

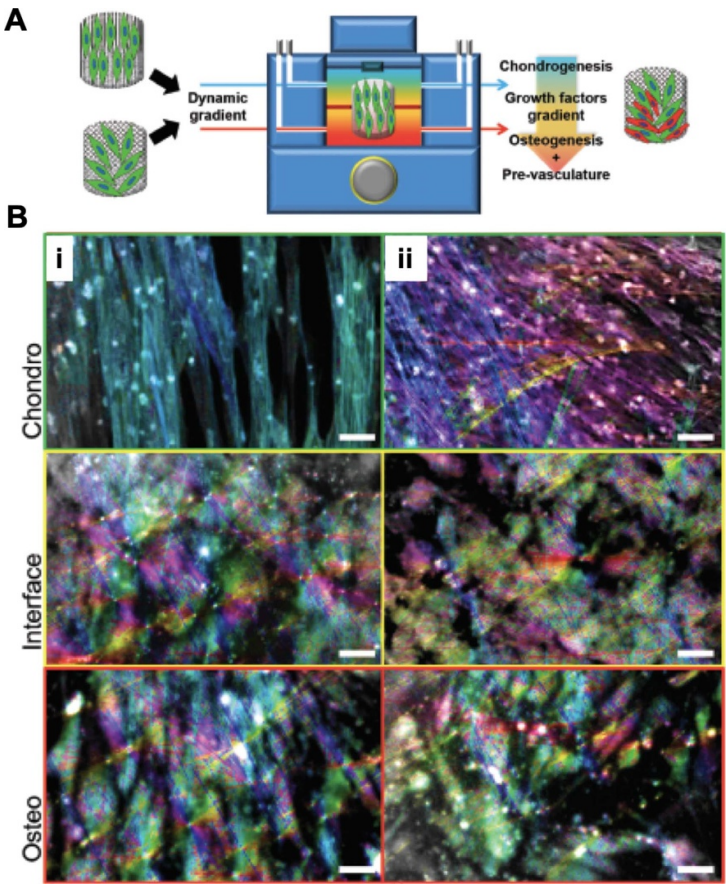

Figure 6. Schematic representation of the 3D porous structures of linear and random porosity. (A) Flow perfusion bioreactor device used to co-culture fat pad hASCs in the 3D anisotropic and isotropic gradient constructs combining the HAp gradient with growth factor cues from culture media. (B) ASCs seeded in: (i) anisotropic, and (ii) isotropic structures and the resulting constructs cultured for 21 d. [15] John Wiley & Sons. © 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

microfluidic devices to mimic the native tissue and providing spatial and temporal control in a much smaller scale as compared to the bioreactors. As example, a cell-polymer solution of MSCs and agarose was cast against a serpentine micro-mold network to produce a biphasic OC construct of two independent microfluidic networks [128]. Constructs were fluidically connected by two controlled flow-loop but enabled the creation of independent cell differentiation parameters for chondrogenic and osteogenic induction. It was possible to observed different gene expression patters and protein levels (e.g. collagen I, II, X) on the networks presenting different thicknesses. Nevertheless, the combination of bioreactors and microfluidic devices has shown to be promising for OC tissue modeling applications, highlighting the most interesting features of both systems. Lin *et al* [129], proposed a flow perfusion multi-chamber bioreactor connected to a microfluidic base that supplied the OC constructs with both chondrogenic and osteogenic environments. That system is composed by a removable insert placed inside a chamber of a microfluidic plate. When the OC construct was placed inside the insert, two chambers were achieved and supplied by the corresponding chondrogenic and osteogenic media. That system allowed to investigate the OC tissue properties in a representative manner and with faster outcomes revealing distinct properties characteristic of the two tissues, as well as, the networks between them creating a system adapted for the maturation of the interface region and with spatially controlled gradients. Following such strategy, it can be stated that the concept of organ-on-a-chip microfluidics evolved to the bioreactors reality [113].

4. Open issues and challenges

4.1. Modelling

Computational modeling has revolutionized the TE field, with the possibility of anticipating through numerical exploitation the biological, structural and mechanical behavior of cells/tissues driven by external stimuli or scaffolds. Before this paradigm, experimental studies were only based on previous knowledge and

literature that not always provided the most accurate information. OCTE is one of the biomedical fields that has evolved with these mathematical simulators [23]. As example, Zhu *et al* [116], used a computational fluid dynamics (CFD) method to simulate the mass transfer of glucose and TGF-β2 in 3D cell-laden hydrogels using a rotating wall vessel bioreactor. The hASCs encapsulated within 3D chitosan/gelatin hydrogel constructs showed equivalent mass transfer of glucose and TGF-β2 to that observed from the mathematical simulation, and with an efficiency superior to that observed on cultures in static conditions. In a different study, Xue *et al* [126] proposed an OC culture system based on additive manufactured bilayered scaffolds and a dual-chamber perfusion bioreactor used for co-culturing ATDC5 and MC3T3-E1 cells in dynamic conditions. In parallel, authors developed finite element models (FEM) of the same manufactured scaffolds based on micro-computed tomography images and computer-aided design, showing significant differences in the internal microenvironment the two FEM with effects experienced by cells, i.e. medium flow velocity and mixing in the bioreactor and fluid-induced shear stresses. With such system, the authors were able to show that it is possible to create a desired microenvironment for OCTE and modeling according to patient-specific needs and requirements. Computer modeling was recently applied for numerical simulation of flow patterns inside microfluidic devices designed for chondrogenic applications [130]. The microfluidic system was focused on the synthesis of alginate nanogels loaded with TGF-β3 in order to achieve an optimal release profile of the growth factor during chondrogenic differentiation of MSCs. To better understand the 3D behavior of the medium flow inside the microchannels a CFD model was applied. By means of adjusting the flow rate ration according to the CFD simulation, it was possible to control the physical properties of the microfluidic synthetized nanogels affecting the release of TGF-β3. As a result, a superior chondrogenic differentiation of MSCs was observed thus revealing the promising outcomes of this system for OCTE applications.

In brief, modelling studies can greatly impact the future of OCTE field and help researchers to better manufacture scaffolds (e.g. architecture) with superior cell-modulating functions (e.g. differentiation). It also can provide in depth knowledge on the desired microenvironment and potentially be used as an inexpensive tool for OCTE.

4.2. Clinical translation/regulatory approval

Several OC tissue engineered products were introduced in the market after revealing promising clinical outcomes, including the TruFit® (Smith ans Nephew, USA), the Maioregen® (Finceramic, Italy), or the Agili-C™ (CartiHeal). All of these devices are reported in the literature as being off-the-shelf OC scaffolds composed of bilayered or trilayered polymeric phases and presenting good clinical evaluation for chondral lesions applications. However, inconsistencies were also detected showing no improvements in *in vivo* biological activity in response to some of the products that are still under evaluation [131, 132]. Other examples of commercial products used for the repair of OC defects include the OsseoFit® plug (Cartilage Repair Device; Kensey Nash Corp.), ChondroMimetic™ (TiGenix NV), BST-Cargel® (Piramal Life Sciences), Bioseed®-C (Biotissue Technologies GmbH), Collagraft® (Nuecoll Inc), HYAFF® 11 (Anika Therapeutics), and Gel-One® (Zimmer) [133–138]. Nevertheless, there is much to research and new materials with refined features are constantly being evaluated for clinical translation. The regulatory approval of these products involves a series of steps that may vary according to the type of product, potential risks, and regulatory powers attributed to the agency. Thus, there is a long way until new medical devices, technologies or therapies to be approved by the regulatory agencies and finally available for clinical applications.

The Food and Drug Administration (FDA) agency regulates almost every steps of new drugs development, that goes from drugs testing, manufacturing, labeling, marketing, efficiency, and safety. Nevertheless, that agency applies different regulation steps for cosmetic products, focus on the labeling and safety aspects. Therefore, depending on the products classification different regulation steps may be conducted. Regarding the approval of novel bioactive scaffold materials for TERM, the first aspect to bear in mind is the patentability of the developed product. Then, the regulation steps always start by evaluating products' degradation profile and safety in pre-clinical and clinical studies, which covers almost 30% of the planned costs [139]. For that, it is necessary to ensure that the company responsible for materials production has enough financial support to follow the regulation for good manufacturing practice (GMP) and produce reproducible and medical grade products. Finally, a market study to evaluate the potential of the products, possible competitors and target audience is also necessary [140, 141].

The ongoing and completed clinical trials (with and without reported results) in the United States of America (USA) and Europe involving TE strategies and devices for OC defects repair and regeneration are listed in table 2.

Table 2. Overview of the ongoing and completed clinical trials related to OCTE strategies and devices for tissue repair and regeneration. Information obtained from https://clinicaltrials.gov.

NCT number	Study title	Intervention/treatment	Study type	First posted-last update	Status	Location
NCT03036878	ReNu™ marrow stimulation Augmentation	Injection of ReNu™ allograft into the joint capsule for the augmentation of marrow stimulation.	Interventional	2017–2020	Completed	USA
NCT03452098	Post-operative rehabilitation of knee osteochondral defect: a case series.	Osteochondral lesions treated with osteoarticular transfer system (OATS) and/or microfracture procedure; physical therapy performed after surgery.	Observational	2014–2017	Completed	USA
NCT02503228	Clinical assessment of the Missouri osteochondral allograft preservation system—MOPS.	Patients with femoral condyle osteochondral defects receiving MOPS-preserved cartilage.	Interventional	2015-Ongoing	Active-Not recruiting	USA
NCT00984594	Evaluation of a composite cancellous and demineralized bone plug (CR-plug) for repair of knee osteochondral defects.	Placement of cancellous and demineralized bone plug (CR plug) autograft in primary defect sites; CR-plug placed in the harvest site.	Interventional	2009–2014	Completedb (has results)	USA
NCT03588975	A Study of MACI in patients aged 10–17 years with symptomatic chondral or osteochondral defects of the knee.	Patients treatment with MAIC (autologous cultured chondrocytes on a porcine collagen membrane) vs arthroscopic microfracture treatment.	Interventional	2018–2019	Recruiting	USA
NCT03299959	Agili-C™ implant performance evaluation.	Patients treatment with Agili-C implantation vs microfracture treatment and/or debridement.	Interventional	2017–2020	Active-not recruiting	USA, Italy, Poland, Belgium
NCT00793104	Evaluation of the CR plug (allograft) for the treatment of a cartilage injury in the knee.	Placement of cancellous and demineralized bone plug (CR plug) allograft in primary injury site; Core defect and implant the allograft CR plug.	Interventional	2008–2013	Completed (has results)	USA

(Continued.)

Table 2. (Continued.)

NCT number	Study title	Intervention/treatment	Study type	First posted-last update	Status	Location
NCT00821873	Evaluation of the CR plug for repair of defects created at the harvest site from an autograft in the knee.	Plug made out of human bone (CR-plug) used to repair a harvest site defect left during OATS procedure.	Interventional	2009–2013	Completed (has results)	USA
NCT03625180	An observational, prospective study of patients with chondral and/or osteochondral defects of the knee treated with NAMIC.	Patients treatment using NAMIC (nanofractured autologous matrix-induced chondrogenesis) technique.	Observational	2018–2018	Recruiting	Spain
NCT04236492	Study protocol to evaluate clinical and imaging results of knee fresh osteochondral allografts.	Transplantation of a fresh osteochondral allograft in the knee.	Observational	2020–2020	Recruiting	Spain
NCT02309957	EAGLE European Post Market Study.	Patients receiving BioMatrix CRD device to repair an articular cartilage lesion or osteochondral defect; BioMatrix CRD™ is a sterile, biphasic, bioresorbable scaffold.	Observational	2014–2019	Completed	Italy, Germany, Netherlands
NCT03908931	Descriptive study of the reconstruction of osteochondral lesions of the knee: clinical and imaging results.	Follow up of osteochondral surgeries by autologous cartilage reconstructions or collagen matrix of the knee using MRI imaging.	Interventional	2019–2019	Recruiting	France
NCT04297449	Prospective 2 year data collection of the first 10 patients after ankle spacer implantation.	Patients with multiple and/or large talar OC defects and patients with failed previous surgical treatments or OA of the talus.	Observational	2020–2020	Recruiting	Germany

EAGLE: evaluation of an acellular osteochondral graft for cartilage lesions; MRI: magnetic resonance imaging.

5. Conclusions

The high level of interfacial OC tissue organization and complexity is now more well-known, as well as, the recognition that the heterogeneous behavior of the tissue is related to gradient properties including structural, mechanical, biochemical, and metabolic. These features are highly dependent, meaning that changes in one of the properties may affect other aspects of tissue performance. Fortunately, the field of OCTE is fast-evolving and researchers have been paying attention to the natural gradients that compose OC tissue. This review aims to address the existing gradients in OC tissue and the recent developments in generated scaffolds and approaches capable to respond to these properties. The applied areas of biomaterials development and processing, additive manufacturing and custom-tailored bioreactors are explored. The use of biologicals has also shown a great promise in developing gradient scaffolds for OC regeneration. Despite it is now becoming possible to develop personalized approaches, i.e. respecting the patient anatomy, some challenges still need to be addressed in order to better mimic the native organization of the OC complex tissue and improve the overall biofunctionality of the newly formed tissue. Thus, further multidisciplinary research efforts need to be performed and the development times should be shortened. In a near future, we certainly will assist to an increasing of research efforts involving computational modelling and *in vitro* models as technological platforms for the *ex vivo* testing of drugs and medical devices applied in the treatment of OC lesions. These show great promise in helping translate faster the novel OC repair/regenerative solutions to the clinics.

Data availability statement

No new data were created or analysed in this study.

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