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Chapter

Therapeutic Potential of Articular Cartilage Regeneration using Tissue Engineering Based on Multiphase Designs

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Abstract

Articular cartilage tissue possesses poor ability to regenerate; as the lesion progresses, it extends to the underlying subchondral bone and an osteochondral (OC) defect appears complicating the therapeutic approaches. Cartilage tissue engineering has become a very active research area capable of contributing to medical technology innovation. In this regard, the development of new biomaterials in combination with cells represents one of the best alternatives for the treatment of OC injuries. In the last decades, the strategies have been designed without considering the cartilage as a complex tissue with a functionally stratified three-dimensional structure. Today, efforts are focused on creating a starting point in the process of cartilage formation with the development of a multiphase implants that recapitulates the cartilage as an OC unit, which improves its integration. This chapter will focus on a review of tissue engineering based on multiphase designs for cartilage and OC injuries, highlighting the importance of the biomaterial selection, and also the relevance of a biomimetic approach to reach a suitable microenvironment for the differentiation and maturation of the chondral tissue.

Keywords: osteochondral regeneration, cartilage tissue engineering, multiphasic designs, biofunctionalization, vascularization

1. Introduction

Clinically, an osteochondral trauma injury usually occurs in the part of the load-bearing of the knee and ankle joint. In the particular case of the knee, as a result, in most animal models, osteochondral defects are created in the femoral condyles (**Figure 1**), which are subject to various types of mechanical loading, such as compression, shear, and hydrostatic pressure. It is commonly accepted that critical size osteochondral defects can induce significant degenerative changes in surrounding cartilage and bone, possibly due to mechanical destabilization that originates from the region of the defect that cannot support the load [1]. In this sense and due to the intrinsic properties of the chondral tissue, the repair of osteochondral defects

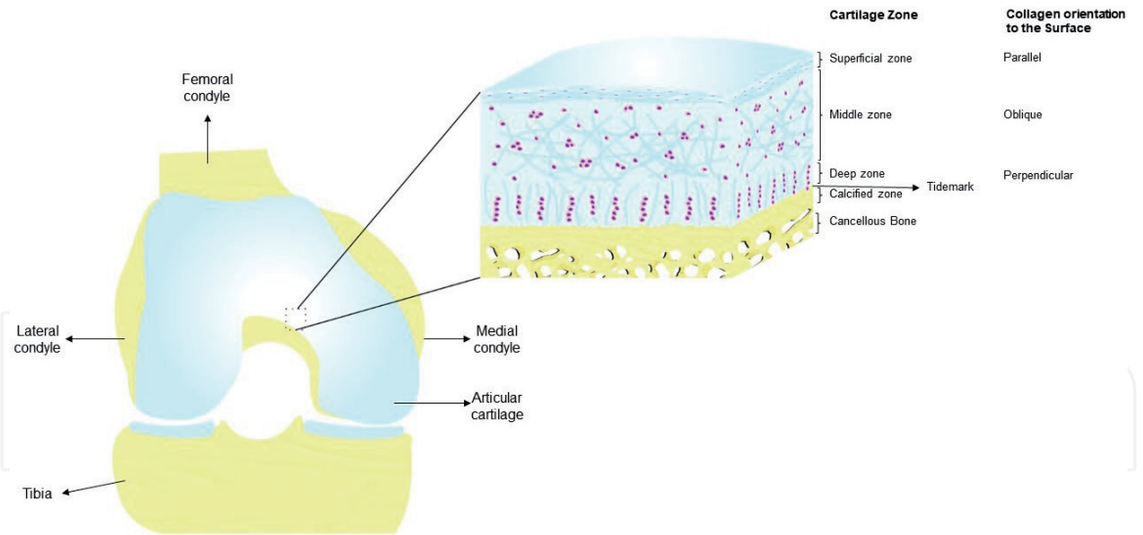

Figure 1.
Hierarchical architecture of the osteochondral unit. The layers including superficial cartilage, middle calcified cartilage, and deep subchondral bone, as well as cancellous bone, are showing; also the orientation of the collagen fibers that give cartilage its resistance compression forces.

requires an approach based on tissue engineering, so that the resulting tissue can mimic the physiological and structural properties of two different tissues (cartilage and bone) by designing specific scaffold-cell constructs. Multiphase approaches use two or three architectures, materials, and even cell types to produce a multilayer construction.

The multiphasic scaffolds have been designed to influence not only the recapitulation of the osteochondral architecture but also to make the integration of the implant with the surrounding tissue more efficient.

In the design of this type of multiphase implants, the selection of bioactive biopolymers and ceramics, but also the manufacturing method, and the dependence or not of the cellularization of the phases in harmony with the presence of the signaling factors will define the therapeutic success. This chapter aims to present and discuss the approaches currently proposed for the use of multiphase designs in the treatment of chondral and osteochondral lesions.

2. The osteochondral unit

Cartilage is a type of connective tissue whose function is to protect the bones of the diarthrodial joints from the frictional forces associated with the load and impact support [2]. Articular cartilage is predominantly avascular, aneural and alymphatic, so the main route of nutrition is through the synovial fluid and assisted by mechanical compression forces [3]. It has a variable thickness according to its location in the body; in humans, it varies from 1 to 3 mm depending on the joint. This tissue is capable of being deformed to increase the total contact surface with the consequent reduction in tension and increase the resistance to damage caused by the applied loads. This function depends on the organization of the macromolecules in the extracellular matrix, particularly the arrangement and orientation of the collagen fibers [4].

The cartilage has a single type of specialized cells called chondrocytes [5], which are embedded and grouped in the extracellular matrix (ECM) secreted by themselves. The ECM is a dynamic network of self-assembled macromolecules composed of water, gases, metabolites, cations and collagen predominantly, noncollagenous glycoproteins, hyaluronate and proteoglycans are also present. The ECM is able to

regulate the behavior of cells and influences their processes of proliferation and maturation [6].

As part of the ECM, water has the function of allowing the deformation of the cartilage in response to stress; it is also important for the nourishment of the cartilage and the lubrication of the joints. Moreover, the capability of the articular cartilage to tolerate significant loads depends on the frictional resistance to water flow and the pressurization of water within the matrix. When the amount of water increases to 90%, as in osteoarthritis (OA), it causes greater permeability, which in turn causes a decrease in resistance and compromises elastic abilities [6].

The most abundant macromolecule in the ECM is collagen and represents 60% of the dry weight of the cartilage. The types of collagen present in the cartilage are I, II, IV, V, VI, IX, and XI; however, type II collagen represents 90–95% of the total amount. Collagen X, on the other hand, is only present in osteochondral ossification phases and, therefore, is associated with cartilage calcification [7].

Proteoglycans (PGs) represent 10–15% of the ECM and are the main noncollagen proteins present in the cartilage. These macromolecules are responsible for the compression of cartilage. PGs are composed of one or more linear glycosaminoglycan chains (GAGs) covalently linked [8].

At this point, we have reviewed the cellular and molecular components of joint tissue, but how are they connected to each other? The articular cartilage has a complex microarchitecture that varies from the articular surface to the subchondral bone, organized into the osteochondral unit (**Figure 1**).

The structure of the osteochondral unit is divided into four well-defined zones designated according to their morphological characteristics, that is, the content of proteoglycans or water and the density of chondrocytes in: superficial, the middle, the deep and the calcified zones (**Figure 1**). In particular, if the differences in the fibrous structure are understood, the terms "tangential," "isotropic," and "radial" have been used frequently. In consequence, the space between these zones allows, identifying three regions: the pericellular, the territorial, and the interterritorial region.

3. Histology and mechanical properties of the osteochondral unit

Each of these zones has a particular matrix composition, and cell morphology, which translates into different cellular, mechanical, and metabolic properties. It is difficult to separate the histological from the biomechanical when the cartilage is analyzed. The particular properties of loading and lubrication of articular cartilage is due, in part, to its composition, which includes a solid phase of collagen fibrils and proteoglycans entangled with a fluid phase [9]. The high tensile stiffness of the collagen considerably increases the compressive strength of the cartilage by also providing resistance to lateral expansion and allowing pressurization of the interstitial water [10]. It is believed that fluid pressurization is an important reason why articular cartilage exhibits a very low coefficient of friction [11].

As heterogeneous material consisting of surface calcified superficial layers (10–20%), medium (40–60%), and deep (30%) and thin. Each layer has specific mechanical properties and is identified by different variations in the size and direction of the collagen fibers. The content of proteoglycans is lower in the surface area and increases with depth.

The superficial area is thin and protects the deeper layers of the shear stresses. It is mainly composed of collagen types II and IX hermetically packed and in parallel alignment with the articular surface. It contains flattened chondrocytes, which are influenced by synovial fluid. This area is responsible for the traction properties of cartilage (**Figure 1**).

Below the surface area is the middle (transition) zone, which represents a bridge between the surface and deep zones. This zone contains a low density of spherical chondrocytes, proteoglycans, and fibrils of thicker collagen and is responsible for resistance to compression forces. The middle zone of the cartilage has looser collagen fibers, which gives it the greater Young compression modulus. It is these variations in tissue morphology that account for the tensile and shear strength properties of cartilage [12] (**Figure 1**).

The deep zone provides the greatest resistance to compression forces. It is formed of larger diameter collagen fibrils in a radial arrangement and a low amount of water. The chondrocytes are organized in a columnar orientation, parallel to the collagen fibers and perpendicular to the articular line (**Figure 1**).

Lastly, the calcified layer of hypertrophic chondrocytes joins the cartilage to the bone by anchoring the collagen fibrils from the deep zone of the subchondral bone (**Figure 1**) [13, 14].

Through the correlation between histology and mechanical properties, it is clear that the collagen network and the proteoglycan matrix within the articular cartilage play an important role in the control of the tensions around the chondrocytes, and in the maintenance of the good condition of the diarthrodial joints when regulating the biosynthesis of the solid matrix.

The effect of the collagen network and the fixed loading densities of the cartilage in the mechanical environment of the chondrocytes have been investigated in a depth-dependent manner. The current model emphasizes that the orientation of the collagen and the negative fixed charge densities dependent on the depth of the articular cartilage have a great effect on the modulation of the mechanical environment in the vicinity of the chondrocytes.

Apart from the structure, the composition of the cartilage is also important to determine the biomechanical properties of the tissue (e.g., traction, compression, and shearing). As mentioned above, collagen fibrils are the main contributors to the traction properties of articular cartilage. Since the different zones have different diameters of collagen and organization, the tensile properties vary significantly between the zones.

4. Clinical strategies for the osteochondral therapeutic approach

The injuries in the articular cartilage are able to stimulate a significant musculoskeletal morbidity not only in elderly patients but also in young people.

The restoration of damage from joint injuries to date represents a great challenge for medicine, since it cannot regenerate spontaneously; moreover, over time it can also lead to the establishment of osteoarthritis (OA).

The classification of articular cartilage injury is performed by instrumented palpation of the lesion and by direct observation by arthroscopy [15, 16]. The most complete classification system is established by the International Cartilage Repair Society (ICRS) [17]. The ICRS grading system evaluates the depth of the lesion and the degree to which the subchondral bone is involved to classify the injury as follows: grade 0 corresponds to a normal joint; grade 1 is presented by superficial lesions, soft cleft, and/or superficial fissures and cracks; grade 2 for abnormal lesions that extend to <50% of the depth of the cartilage; grade 3 due to serious abnormalities in which cartilage defects extend to >50% of the depth of the cartilage, as well as to the calcified layer and up to, but not through, the subchondral bone; and grade 4 for severe abnormal where there is also development of blisters in the tissue [17].

Articular cartilage has a limited capacity for repair. Injured chondrocytes (either superficial or partial thickness lesions) from the early stages develop

defects in their metabolism; therefore, they are unable to maintain a normal concentration of PGs [18].

These modifications trigger the increase in tissue hydration and therefore the fibrillar disorganization of collagen [3, 19]. These changes favor an increase in the transmission of force toward the subchondral bone. By exceeding the capacity of the subchondral bone, the impact on the damaged cartilage is even deeper.

In response to this series of events, the chondrocytes proliferate and therefore the production of matrix molecules at the area of the lesion increases, however, the new matrix is not able to restore the native surface [3].

When the lesion reaches the subchondral bone (full-thickness lesions), the entry of pluripotent medullary elements is observed [20]. These migratory mesenchymal stem cells produce type I collagen fibers to fill the full thickness defect with fibrocartilage. It should be noted that fibrocartilage is not capable of supplying the damping functions of articular cartilage [21].

Following this line of argumentation, the strategies designed for the treatment of articular cartilage lesions can classically be classified as discussed below.

Palliative as physiotherapy and systemic medications to relieve pain; reparative procedures such as debridement, washing of the knee and ankle joint, arthroscopic arthroplasty, microfracture, and bone marrow stimulation techniques; restorative such as high tibial osteotomy, unicompartmental knee arthroplasty and total knee arthroplasty; and transplantation such as osteochondral transplantation (osteochondral graft), osteochondral autologous transplantation (OATS), and transplantation of a autologous chondrocyte implantation (ACI) [22, 23].

4.1 Microfracture

Classified within the reparative procedures is the microfracture. Microfracture was introduced into the clinic after other techniques of bone marrow stimulation were used in the late 1980s and early 1990s to penetrate the subchondral bone. This technique improves the migration of MSCs from the bone marrow to the site of the cartilage defect; however, microfracture often results in the formation of fibrocartilage that is biochemically and biomechanically inferior to hyaline articular cartilage [24]. A case series study has shown that without the mechanical robustness of the hyaline tissue, the repair tissue is vulnerable to joint mechanical forces and typically deteriorates between 18 and 24 months after surgery. Such deterioration is particularly evident when treating large defects or those located in the patellofemoral joint [25].

Although the FDA and many physicians still consider microfracture to be the gold standard for cartilage repair, prospective comparative studies show that microfracture could delay cartilage degeneration only in the short term; more than 5 years after surgery, treatment failure can be expected regardless of the size of the lesion [26].

4.2 Osteochondral autologous transplantation (OATS)

Osteochondral autologous transplantation has been indicated majorly for small-to-medium size (diameter > 10 mm) focal articular cartilage or osteochondral defects of the weigh-bearing areas of the femoral condyles, patellofemoral joint and talus without an acceptable outcome after less invasive techniques [27].

In OATS, a single or multiple osteochondral grafts are harvested from either the less-weight-bearing parts of the femoral condyle or the costal-osteochondral junction. This surgical procedure has the advantage of transplanting viable hyaline cartilage and subchondral bone, which is then transplanted into the defect area to restore the integrity of the articular surface [28].

The disadvantages are basically two: the availability of the grafts and the morbidity of the donor site. The major disadvantage of this procedure is the need to harvest one or multiple grafts from an asymptomatic knee or the rib area. Osteochondral harvesting in OATS often results in considerable donor-site morbidity, showing rates of 17 and 6% for ankle and knee mosaicplasty procedures, respectively, without any significant correlation between the rate of donor-site morbidity and size of the defect, number, and size of the plugs [29]. Furthermore, there is limited evidence on the short- and long-term consequences from harvesting bone plugs of asymptomatic joints.

4.3 Implantation of autologous chondrocytes

The inconsistent results of microfracture opened the way to the development of autologous chondrocyte implantation (ACI). To perform this technique, a sample of cartilage of full thickness is collected from a region of the joint under heavy weight; this by means of a biopsy during a first arthroscopic operation, the biopsy would thus serve to provide a population of chondrocytes that would later be expanded in vitro, to generate around 12–48 million cells. During a second operation, the chondrocytes would implant in the defect of the debrided cartilage to finally be covered with a membrane. This technique has two main benefits: the use of a patient's own cells, which avoids possible complications related to immune events or viral infections when transplanting allogeneic cells or foreign materials, and unlike the autologous osteochondral implantation, the small biopsy minimizes complications in the donor zone of chondrocytes [30].

The positive clinical and functional results of the ACI have been confirmed by clinical trials [31, 32]. The series of long-term cases with 5 years of follow-up have shown that ACI is an effective and durable treatment for knee cartilage lesions greater than 4 cm^2 [33, 34].

It should be noted, however, that the ACI has three main drawbacks:

- Two operations are needed; this makes the recovery time very long (6–12 months) to guarantee the maturation of the neoformed tissue and thus achieve improved clinical scores from the beginning of the study.

- The most frequently reported adverse event after ACI, using a periosteal flap to seal the cells implanted in the cartilage defect, is flap hypertrophy [33]. Therefore, alternative approaches use artificial matrices such as porcine membranes consisting of collagen mixtures types I and III or hyaluronic acid scaffolds [34–36]. These materials eventually increase the likelihood of an immune reaction, and their use is currently considered not approved in the United States.

- Preliminary studies have also shown that very often, autologous chondrocytes are "dedifferentiated" to fibrochondrocytes in culture [37]. Although other studies show that they can be redifferentiated and express chondrocytic markers after being reintroduced in an *in vitro* 3D culture system [38], large-scale cohort studies are needed to continue investigating the cost-effectiveness of the ACI in this regard.

4.4 Scaffolding-based techniques

Taking into consideration the systems that allow the grafted chondrocytes to be embedded in a three-dimensional system (3D), the osteotomy and autologous osteochondral graft transplantation has been suggested to restore normal joint congruity and minimize joint deterioration. Often, these techniques have not

resulted in long-term a clinical solution, which has prompted the development of approaches that involve regenerative medicine and tissue engineering to restore articular cartilage.

The lack of a support material or scaffold to guide the synthesis and organization of the neoformed ECM could, in part, explain the variability of the results among the populations of patients treated with ACI techniques. *Ex vivo* studies have shown that successful regeneration of cartilage depends on both the proliferation rate of chondrocytes and the differentiation capacity of stem cells within a three-dimensional scaffold designed by tissue engineering; this structure then acts not only as a vehicle or cellular support but also influences the properties especially the mechanical properties of the newly formed tissue [39].

5. Tissue engineering based on multiphase designs for cartilage regeneration

Tissue engineering can be defined as the creation or induction of the formation of a specific tissue, in a specific location, through the manipulation and selection of cells, matrices, and biological stimuli. It is an interdisciplinary field that applies principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function [40].

Currently, tissue engineering combines the contribution of cells, undifferentiated or not, which are placed into a scaffold where growth factors can be added to accelerate cell proliferation and differentiation so that after being transplanted to a damaged structure and reaching its regeneration.

5.1 Strategies for cartilage tissue engineering

In the cartilage tissue engineering, the constant development of new designs combining biomaterials, with different cellular sources and modifying the cell culture methodology within the scaffolding systems is driven by the need, still not satisfied, to have a gold standard for functional and long-term repair of chondral and osteochondral defects.

As a cellular source for the formation of cartilage, chondrocytes, or alternatively, mesenchymal stem cells can be used. In the case of mesenchymal stem cells, there are a series of known factors that induce their differentiation toward the chondral phenotype, among which are the use of a culture medium without serum, enriched with dexamethasone, ascorbate, TGFβ, and BMPs, being the method of three-dimensional cultivation at high density one of the most used for this purpose [41, 42].

For the implantation of cells in the cartilage defect, they should be embedded in the thickness of scaffolding. These cellularized graft needs to be maintained for some period of time in culture in order to allow the cells to secrete enough ECM to functionally replace the normal cartilage and facilitate its complete integration. A newly developed osteochondral construction with inferior mechanical properties can also contribute to mechanical imbalance near the defect region until its mechanical properties have matured [43]. Mayr et al. demonstrated that the cartilage component of the osteochondral graft had only half the rigidity of the surrounding cartilage 6 months after implantation [44]. The longer the osteochondral graft takes to mature into mechanical properties, the longer the surrounding cartilage will be exposed to an excessive load, which may contribute to degenerative processes. Therefore, it is necessary to select scaffolds that allow building structures related to the biological behavior of cells into an adequate environment. In any case, for the production of cartilage, it is important to achieve

the maximum production of extracellular matrix because the mechanical behavior of the implanted artificial tissue is favored, especially when using less resistant scaffolds than the normal articular cartilage.

The application of cells in scaffolds, as tissue engineering does, makes cartilage regeneration strategies complex but allows the process to be orchestrated efficiently. The critical point in these strategies is the expansion of the cells in culture in order to generate a suitable production of ECM *in vitro* and with a supportive impact on the mechanical properties *in vivo* [45].

Another challenge to overcome regarding this strategy is to achieve a competent integration of the graft after implantation. The integration of the implanted tissue with the organ requires remodeling, degradation, and at the end, formation of new tissue. The remodeling of the implanted tissue is essential for its functionality [45].

In the last decades, the strategies have been designed without considering the cartilage as a complex tissue with a functionally stratified three-dimensional structure. Today, efforts are focused on achieving a benchmark in the cartilage formation process with the development of a multiphasic implant, not only because it recapitulates the nature of native tissue, but also it takes advantage of the healing capability of bone to promote the implant integration with the surrounding tissue and then bone healing and cartilage formation. The architecture of the scaffold and the presence of migratory cells within or immediately around the graft in the bone phase of the osteochondral tissue then play a key role in the integration and therefore tissue repair.

5.2 Multiphasic scaffolding

During the last decade, there have been many new developments in various aspects of scaffolding manufacturing. Computer-aided designs and fabrication technologies are used to fabricate custom scaffolds for irregularly shaped defects [46, 47].

The materials used for scaffolds and matrices are increasingly intelligent and more versatile, and can be modified to incorporate bioactive peptides [48]. Although scaffold fabrication technologies are advancing at a rapid pace, no engineering strategy used to date can completely recapitulate the biochemical and physical characteristics of native osteochondral tissue. Although it is generally useful to simplify the approach of *in vivo* repair from an engineering point of view, for a successful *in vivo* result, the biological complexities that take place within the joint must also be taken into account in the design.

The osteochondral tissue has a heterogeneous multilayer structure composed of uncalcified cartilage (superficial, middle, and deep zone), calcified cartilage and subchondral bone.

Essentially, a multiphasic scaffold should be biocompatible able to guide the structuring of new chondral and osseous tissue, taking into account the presence and biological functionality of the interface region between them (tidemark) to achieve the mechanical properties of articular cartilage. The widespread approach uses multicomponent systems, and the exquisite melding of natural and synthetic biomaterials where the assembly strategy is fundamental since it determines the topography and the structural arrangement in which the extracellular matrix is organized, a random or a well-ordered orientation of the fibers within the chondral phase in particular.

It can be postulated that the typical lack of orientation of the collagen fiber in the repaired cartilage also has a role in the prevention of a strong integration at the level of the cartilage. The surface area of the cartilage in the normal cartilage is horizontally aligned, parallel to the direction of the joint. However, within the repaired cartilage, this provision is often lacking; therefore, the border adjacent to the native

cartilage tissue and modified by genetic engineering is susceptible to rupture. The vertical orientation of collagens near the subchondral bone has been attributed to the anchoring of cartilage tissue against large strains [49]. The lack of orientation of the collagen in a dynamic loading environment of the joint probably has a role in the *in vivo* failure of the implanted constructions and the decreased integration.

Additionally, it is well documented that the rigidity of the cartilage depends on the depth, and that the superficial layer of the cartilage deforms much more than the deeper layers [50]. In this respect, when the cartilaginous component of the osteochondral scaffolds lacks the deformation patterns that vary in depth, it is likely that the levels of compression deformation mismatched between the cartilage and the implant cause a higher shear stress in the interfacial region, causing a break. Tissue engineering cartilage grafts with variable depth compressive properties have also been proposed in the past [51], and can be incorporated into future osteochondral designs.

Multiphasic scaffolds can be designed considering two or three different phases (biphasic or three phase, respectively), each of them with an architecture and composition of particular biomaterials. Since the cartilage and the subchondral bone, part of the osteochondral unit, have different biological and mechanical requirements, the first approaches in the design of multiphase implants were based on the use of two different biomaterials in order to reach a tissue-specific scaffold design; moreover, the use of different combinations of biomaterials for each phase has been reported.

Polylactic-acid (PLA)-coated polyglycolic acid (PGA) scaffold molded by the computer-aided design and manufacturing (CAD/CAM) technology proved to be ideal scaffolds for cartilage regeneration, where the presence of PLA provides adequate rigidity for the chondral phase, which is attached to polycaprolactone/hydroxyapatite (PCL/HA), an osteoconductive material, where HA generates a favorable topographical surface and biomimetic microenvironment in terms of bone tissue. The regenerated cartilage and subchondral bone showed a well-structured transition zone between the two phases, which has resulted in a better integration with the host and with mechanical properties capable of supporting the solicitation of the chondral tissue [52].

The importance of generating in the scaffold a tissue-specific microenvironment that allows the undifferentiated migrant cells of the host to find a niche for the adequate differentiation toward the chondrocytic lineage is evident.

Yun-Jeong et al. have shown that not only the microenvironment generated by the composition of the biomaterials impact on a better imitation of the osteochondral unit, but also the scaffolding structure has an important influence, being an aligned structure the most adequate in comparison with a randomly structured scaffold [55]. A stratified design of aligned channels in a biphasic scaffold using collagen type I and biphasic calcium phosphate (BCP) to mimic the cartilage and bone tissue, respectively, was manufactured by using an exquisite unidirectional freeze-casting process. Collagen is flexible, and it has specific molecular domains able to induce and support cell bioactivity [53]. Likewise, BCP provides significant osteoconductivity, bioactivity, and mechanical features [54]. However, privileging on the composition, the generation of a biphasic scaffold with a longitudinal roughness of the inner channels that serves as a guide for the correct adhesion of the cells; it results in a topography that truly emulates the osteochondral unit and shows *in vivo* a superior regeneration of the osteochondral tissue compared to the random structure [55].

Therefore, not only the pore size and porosity should be taken into account for a correct design, but also the alignment of the channels within the scaffold influencing cell migration and the proper pattern fibers of the ECM.

Likewise, multiphase can be assembled on the basis of a single biomaterial. It is possible to modify the physical properties such as roughness, pore size, and

interconnectivity particularizing according to the phase, selecting a specific type of porogen and particle size, as well as through the use of different solvents and the polymerization process.

Biphasic scaffold with a cartilage phase consisting of a silk scaffold attached to a bone phase consisting of a strontium-hardystonite-gahnite (SHG)-silk scaffold has been designed by Jiao Li et al. [56]. The preparation implies a coating process of SHG ceramic scaffolds with a single silk layer using an aqueous silk fibroin solution then attached to the mixture of silk using methanol as an alternative solvent prior to silk scaffold formation in order to induce β-sheet formation in fibroin and the structure of the interface. The conformation of this design showed not only the ability to promote the differentiation of human mesenchymal stem cells toward the chondrogenic or osteogenic lineage, but also in addition, by having a well-stratified biphasic structure, the loading behavior validated the compression properties.

By the same token, a single biomaterial can be used and can generate distinct microenvironment using different molecules to biofunctionalize in a tissue-specific manner. Certainly, no biomaterial is intrinsically capable of satisfying all the requirements for the manufacture of complex and stratified tissues, so the biofunctionalization of these is presented as an alternative procedure to adapt the properties of the biomaterials to the needs of the chondral or bone tissue.

A biphasic, but monolithic scaffolds based on alginate, a highly biocompatible natural biomaterial able to support the growth of diverse cell lineages is designed by Schütz et al. through its strategy; scaffolds are fabricated by a diffusion-controlled system that allows the directed ionotropic gelation [57]. The final structure leads to the formation of channel-like, parallel aligned pores. In order to generate a chondral environment, alginate is biofunctionalized with hyaluronic acid, while for the bone phase, hydroxyapatite is used.

This simple procedure generates two well-defined layers characterized by different microstructure and mechanical properties, which provide a suitable environment for cells to form the respective tissue. Although an interface between the chondral and bone areas of the implant is not structured, a stable connection between them is clearly demonstrated, which positively impacts the mechanical properties in the final design. According to the influence of biofunctionalization, it was demonstrated by gene expression analysis that the embedded stem cells differentiated into the chondrogenic lineage when they were cultivated in chondrogenic medium; additionally, under the stimulation of the hyaluronic acid present in this phase, the chondrocyte phenotype remained stable.

Biofunctionalization, especially for monolithic scaffolds, is a useful alternative to provide chondro- and osteoinductive properties. Aragonite is a biomaterial from coral exoskeletons, similar to human bone including its 3D structure and pore interconnections as well as its crystalline form of calcium carbonate ($CaCO_3$) [58]. That features confers improved osteoconductive ability, suitable for bone regeneration.

Interestingly, specific coral species differ in size and interconnectivity of coral pores, which expands the range of applications for different tissues. In order to induce chondrogenesis in a monolithic system of aragonite, the use of hyaluronic acid has been described by Korn et al. [59]. We have already discussed before, the relevance of channel generation aligned in parallel to guide the adhesion of the cells in the chondral phase and the subsequent structuring of the ECM. In this design, in addition to the biofunctionalization with hyaluronic acid, the mechanical modification of drilled channels is also added. The combination of the two strategies showed in a model of joint damage in goat the best results compared to aragonite alone, and in the absence of parallel

channels; it means a cartilaginous repair tissue with hyaline cartilage as shown by the marked expression of proteoglycans, as well as of collagen type II and absence of collagen type I.

6. Influence of vascularization on scaffold design for osteochondral regeneration

Vascularization is the bottleneck in tissue engineering. Creating constructs in the laboratory that lack of the proper vessels network will fail after implantation as the cells will not get enough oxygen and nutrients and will die. This fact is even more significant for osteochondral regeneration. Bone is a highly vascularized tissue while cartilage is avascular. When vascular networks invade cartilage surface from the subchondral zone, it might lead to an ossification of the cartilage from the deep and intermediate zone implying a joint damage and increasing pain. The design of the optimal scaffold to control angiogenesis, promoting vessel growth from preexisting ones, on the bone side and inhibiting it on the cartilage side is relevant for osteochondral regeneration.

One strategy to improve bone formation is to use growth factors (GFs) that can activate angiogenesis within the scaffold. There are several GFs involved in angiogenesis, such as vascular endothelial growth factor (VEGF), platelet-derived GF (PDGF), bone morphogenic proteins (BMPs), fibroblasts growth factors (FGFs), and tumor growth factor beta (TGFβ) [60]. Uploading VEGF is widely used, as the VEGF activates endothelial precursor cell (EPC) migration and proliferation activating the angiogenic process, and subsequently promotes the recruitment and survival of bone forming cells improving bone regeneration. However, the presence of high levels of VEGF is one of the factors related to OA progression, inducing cartilage degeneration and pain [61].

Therefore, the scaffold design for osteochondral regeneration must fulfill different properties that are shared by the two tissues, such as cell adhesion and proliferation and a high production of ECM; but others must deal with angiogenic promotion for bone or angiogenic inhibition for cartilage. Furthermore, the already observed side effects of supraphysiological doses of bone-related GFs heterotopic bone growth, pseudoarthrosis, local inflammation, and immune response [62] must be controlled by means of delivery vehicles that will ensure the bioactivity of these molecules and the remaining in the target location over the therapeutic timeframe. This can be done by covalent attachment to the scaffold, noncovalent binding, or with the nanoparticle carriers.

Kempen et al. developed a system for the sequential release of VEGF with BMP-2. BMP-2-loaded PLGA microspheres in a poly(propylene fumarate) (PPF)-scaffold combined with a VEGF-loaded gelatin hydrogel in a rat subcutaneous model demonstrated both improved vessel and bone formation when compared to scaffolds that did not contain VEGF [63].

García-Fernández et al. used antiangiogenic polymer based on 2-acrylamido-2-methylpropane sulfonic acid (AMPS) and a methacrylic derivative of 5-amino-2-naphthalenesulfonic acid (MANSA) [64]. The use of this synthetic polymer completely inhibited angiogenesis by the interaction of the sulfonic acid groups with the bFGF and VEGF modulating their activity in the processes of endothelial cell migration and proliferation. Thus, the fabrication of a biphasic scaffold by combining two different polymers that can control angiogenesis can be an efficient approach.

An innovative approach that has been tested recently is the use of microRNAs(miRNAs) to modulate cell activity for regenerative medicine applications. miRNA is a single-stranded RNA, with a length between 21 and 25 nucleotides that can regulate gene expression, usually by destabilizing mRNAs or

by suppressing translation. The roles of these miRNAs on bone diseases (such as osteoporosis, osteoarthritis, and rheumatoid arthritis) have been recently highlighted. Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures [65, 66]. miR-26a was reported to regulate angiogenesis by targeting BMP/SMAD1 signaling in endothelial cells [67].

The use of these molecules as miRNA regulators can be done by using synthetic molecules, which either mimic or repress the function of endogenous miRNAs. The mimicking molecules will enhance the suppression of the target protein synthesis by degrading the miRNA or inhibiting the protein translation. On the other hand, miRNAs inhibitors (antagomirs) preventing the activity inside the cells will lead to a rise of mRNA and protein expression. This approach can be used to upload scaffolds with either agonist or antagonist molecules to induce or avoid vascularization.

Many scaffolds have been designed to fulfill the function of miRNA delivery, mainly hydrogels, nanofibers, and porous or spongy scaffolds. Besides, the normal desired properties such as biocompatibility, easy fabrication, easy sterilization, proper mechanical properties, and adequate porosity for vessels growth, the material must retain the miRNA complexes while facilitating their sufficient exposure to the infiltrating cells without affecting its mechanical properties [68].

7. Biomaterials for multiphasic scaffolding

Biomaterial scaffold properties are fundamental to guide and recreate the native environment. The biomaterials for osteochondral applications in first insight must be biocompatible and should be intrinsically osteoinductive, osteoconductive, chondroinductive, or chondroconductive, and not less insignificant, and must possess a degradation rate that allows the formation of new tissue.

As previously stated, an ideal scaffold for the treatment from a multiphase point of view must have a chondrogenic matrix that is flexible, resistant and with pores small enough to mimic the hyaline cartilaginous matrix and an osteogenic matrix that should be mechanically competent similar to cancellous bone and bioactive, which has larger pores that mimic the microenvironment of the subchondral bone.

Achieving an articular cartilage design capable of mimicking its anisotropic mechanical behavior, still represents one of the greatest challenges in the cartilage tissues engineering [69]. In addition, the ideal biomaterial for cartilage should allow the cartilage composition to be recreated in terms of the liquid and solid phases of the connective tissue, reproduce its zonal organization, and facilitate the integration of the neoformed tissue with the adjacent native tissue.

Functionally, we can classify biomaterials into: protein-based polymers, such as fibrin, gelatin, collagen, and silk fibroin [70–74]. Biopolymers based on carbohydrates such as alginate, chitosan, agarose and polyethylene glycol [75–78], and synthetic polymers such as polylactic acid, polyglycolic acid, polycaprolactone and polylactic-coglycolicacid (PLGA) are the most common [79–81].

7.1 Carbohydrate-based polymers

These kinds of biomaterials are comprised of cross-linked polymers that swallow a great amount of water, which empathizes with the features of cartilage ECM, thus favoring the maintenance of spherical morphology within the scaffold [76]. Furthermore, synthetic materials and growth factors can be added in order to enhance chondrogenesis.

A material with adequate characteristics for cartilage engineering is chitosan, a polycationic polysaccharide that can be degraded enzymatically by the lysozyme

present in the MEC of human cartilage. Chitosan has a chemical similarity with GAGs, which gives it the ability to interact with them [82]; through various *in vitro* studies, it has been demonstrated that scaffolds based on chitosan especially in combination with other biomaterials such as collagen II [108], hyaluronic acid [83], or fibroin [84] promote chondrogenic activity and support the production of aggrecan and type II collagen, thus improving cartilage repair [108].

7.2 Protein-based polymers

Among the materials of a protein nature is gelatin, which is formed from denatured collagen and can bind to growth factors, proteins, and peptides and is also capable of promoting efficient cell adhesion. On the other hand, there is the collagen that constitutes the main structural component of the ECM, and its use as a scaffolding material allows the cells to retain their phenotypes [85].

Collagen is a naturally occurring protein found in various fibrous tissues such as bone and cartilage. Collagen-based scaffolds have been used for cartilage tissue engineering applications as biomaterials due to its biocompatibility and biodegradability. Type I collagen gels seeded with bone marrow-derived MSCs have shown the formation of cartilage and subchondral bone after implantation in a full-thickness osteochondral defects macaque model. After 24 weeks, the defect had been covered with cartilage-rich reparative tissue, suitable integration with the surrounding cartilage tissue, and restoration of trabecular subchondral bone [86].

As part of this group of biomaterials is the silkworm fibroin, which is a natural biopolymer, with properties such as biocompatibility and biodegradability that allow it to be currently used for the development of a wide variety of biomedical devices and new regeneration technologies [87]. Fibroin is the main constituent (72–81%) of silkworm cocoons *Bombyx mori* [88], is a hydrophobic glycoprotein containing a large amount of hydrogen bonds, its composition and molecular orientation allows the formation of a semicrystalline structure formed by a highly ordered phase of antiparallel β-sheets that give it strength and tenacity, separated by less ordered β-sheet spacers that in turn contribute to the flexibility and elasticity of the fiber [89].

Because of these unique intrinsic properties and their versatility, silk alone is used as a biomaterial for biotechnological processes and as well as in tissue engineering [56, 90, 109]; however, it can also be combined with other polymers; the combination of fibroin/hyaluronic acid is reported, which favors the cultivation of mesenchymal stem cells [91]. In this context, silk fibroin has interesting applications in the engineering of hard and soft tissues and has diverse characteristics among which is included the ability to support the proliferation and differentiation of various cell types, making it an attractive therapeutic candidate in cartilage regenerative medicine (**Table 1**) [56, 109].

Silk fibroin has been used in several medical applications, and it can be used as fiber [92], electrospun fibers [93], films [94], or hydrogels [95]. The versatility of fibroin as a biomaterial makes it suitable for any type of application in tissue engineering, and applications that demonstrate greater maturity and close to its final application are in the field of regeneration of bone, cartilage, and ligaments.

In this regard, a very interesting application is the reconstruction of the cruciate ligament of the knee through the elaboration of a cord of silk fibers that later are sown with mesenchymal cells of the bone marrow that differentiate to ligament tissue, offering a mechanical resistance much superior to that of other organic materials and a great biocompatibility. This application is already in commercial phase in the United States, by a company specialized in the development of biomaterials based on silk fibroin (Serica) [96].

Regarding the regeneration of cartilage, fibroin has been used for the manufacture of biphasic implants in combination with bioactive ceramics or 70S bioactive glass, which

Multiphasic design	In vitro evaluation	Preclinical evaluation	Clinical evidence	References
Bilayered scaffold using microfibrillar articular cartilage extracellular matrix (ACECM) and cellularized with rabbit chondrocytes [chondral phase], attached to ACECM and nanophase hydroxyapatite (HAp) [bone phase]	A gradual interfacial region was formed; for chondral phase, intensely stained with safranin O and toluidine blue, indicating an ECM rich in sulfated proteoglycans, while bone phase a positive alizarin red staining of the lower layers indicated the rich Ca content	Not performed	Not performed	[106]
Juvenile ovine articular chondrocytes (joACs) embedded in agarose [chondral phase], attached to hydroxyapatite (HAp) ceramics [bone phase]	Suitable compressive strength due to HAp. Chondrocytes were densely packed in a GAGs and collagen-rich ECM, showing a zonal organization reminiscent of native cartilage	Not performed	Not performed	[107]
Chondrocyte cultivated on a biphasic, type II collagen–chitosan, attached to poly(lactic-co-glycolic acid) [PLGA] bone scaffold	Efficient integration between chondral and bone phases with suitable pore size differences. In vitro chondrogenic differentiation confirmed by the expression of collagen type II, Sox-9 and a remarkable upregulation of aggrecan	Not performed	Not performed	[108]
Biphasic scaffold with a cartilage phase consisting of a silk scaffold attached to a bone phase consisting of an SHG-silk scaffold (strontium- hardystonite-gahnite), and cellularized with hMSCs from bone marrow	A well-integrated interface with a stratified compressive properties according to osteochondral tissue. The structuring and maturation of the ECM congruent with the distribution and structure of the hyaline cartilage	Not performed	Not performed	[56]
Biphasic scaffold with a cartilage phase consisting of a silk scaffold well integrated to a silk-nanocalcium phosphate as a bone phase, and cellularized with rabbit bone marrow mesenchymal stromal cells	No data are presented	In a rabbit knee critical size osteochondral model. By histological and immunohistochemical analysis, cartilage regeneration and abundant presence of glycosaminoglycan and collagen II were observed. The formation of *de novo* subchondral bone and blood vessels were also observed	Not performed	[109]
A biphasic hydrogel composed by methacrylated chondroitin sulfate (CSMA) as chondral phase, and acryloyl chloride-poly(ε- caprolactone)-poly(ethylene glycol)- poly(ε-caprolactone)-acryloyl chloride (PECDA) as bone phase, with an interface of alginate; and cellularized with chondrocyte and osteoblast, respectively	Strong interfacial bonding and improved mechanical properties; highly interconnected porous structure suitable for cellular adhesion and growth in tissue-specific way	The scaffold induced a very weak inflammatory response and in a rabbit, osteochondral defect model provided a temporary structure and an adequate microenvironment for the ingrowth of osteochondral newly formed tissue	Not performed	[110]

Multiphasic design	In vitro evaluation	Preclinical evaluation	Clinical evidence	References
Biphasic acellular scaffold with a cartilage phase consisting of hyaluronic acid attached to a bone phase consisting of aragonite	Scaffold has an interconnecting porosity, with an average of 100-μm pore size, able to support human stem cell adhesion	Using a goat model of a critical osteochondral defect, the formation of hyaline cartilage and subchondral bone regeneration in the area of the lesion is demonstrated [111]	The scaffold was used on an Outerbridge grade IV promoting the mesenchymal stem-cell migration, and after 24 months, the articular surface appeared restored by MRI (Agili-C™) [112]	[111, 112]
Acellular scaffold made from polylactide-coglycolide copolymer and a bone phase containing calcium sulfate	A porous and resorbable scaffold with an osteoinductive environment due to the added calcium sulfate. The polyglycolic acid fibers, which are arranged in an orderly manner, give the structure a mechanical reinforcement [113]	Using a goat model of a critical osteochondral defect, a good filling of osteochondral defects, suitable integration with the native cartilage, and a high percentage of hyaline-like cartilage were demonstrated [114]	Reports in the literature about TruFit™ are controversial. It has been reported a poor integration of the implant with the surrounding tissue and poor bone regeneration [119, 120]. By contrast, it it has been used on an Outerbridge grade III and IV. Magnetic resonance imaging (MRI) shows an adequate integration of bone scaffolds in studied cases for more than 5 years and a sufficient restoration of the articular cartilage (Trufit™) [115]	[113–115, 119, 120]
Acellular, tri-phasic biomimetic scaffold with a cartilage phase consisting of equine type I collagen, a tidemark layer consisting of type I collagen and magnesiumhydroxyapatite (Mg-HA), attached to the bone phase consisting of a mineralized blend of type I collagen and Mg-HA	No data are presented	By sheep and horse model, the secretion of type II collagen in the cartilage region, and a uniform presence of type I collagen in the subchondral layer were evidenced. Also, the regeneration with good quality and well-integrated tissue was demonstrated [117, 118]	The scaffold has been used on an Outerbridge grade III and IV. MRI was performed and evaluated by magnetic resonance observation of cartilage repair tissue (MOCART) score. All the scores improved significantly from the baseline (Maioregen™) [116]	[116–118]

Table 1.
Multiphasic designs for osteochondral repair.

has allowed the obtaining of scaffolds with stratified properties capable of satisfying the complex and diverse regenerative requirements of the osteochondral tissue [97].

7.3 Synthetic polymer-based biomaterials

Several biodegradable and biocompatible polymers of synthetic origin have been developed for biomedical applications. The aliphatic polyesters, that is, poly (α-hydroxy esters), represent polymers that have great potential for their application in tissue regeneration. In this group are listed: poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and poly (ε-caprolactone) (PCL) [12, 13]. There are three enantiomers of PLA, L-lactide, D-lactide, and mesolactide [98]. Of these, the most used are poly (L-lactide) (PLLA) and poly (D-lactide) (PDLA) [99]. Both PLLA and PDLA have a tensile strength and elongation at break (1–8%) [100, 101], its nature of slow crystallization predisposes that these materials are typically hard and brittle. *In vivo* studies have shown that highly crystalline PLLA degrades completely in 5 years, while mostly amorphous PDLLA loses strength in less than 2 months and is degraded in 1 year [102].

The material properties, degradation rates, and tissue compatibility of PLA can be modified by copolymerization with other monomers, resulting in copolymers such as poly (lactic acid-co-caprolactone) (PLGA), poly (lactic acid-co-caprolactone) (PLCL), poly (lactic acid-co-ethylene glycol) (PLEG), and poly (lactic acid-co-glutamic acid) (PLGM); this makes them biomaterials with highly adaptable properties for broad biomedical applications (**Table 1**) [108, 110, 113–115].

The most common synthetic material used for cartilage tissue engineering has been nonwoven PGA and PLA mesh. PGA has demonstrated good chondrogenesis both *in vitro* and *in vivo* [103]. A combination of a cell-free poly (L-lactic-coglycolic acid) scaffold and in situ bone marrow stem cells has been used for focal full-thickness cartilage defects in a rabbit model, demonstrating suitable integration of the implant and hyaline-like cartilage regeneration in 24 weeks [104].

These polymers have been approved by FDA: a PGA, PLA, and also polydioxanone-based copolymer; BioSeed1, BioTissue Technologies, Freiburg, Germany has been used for hyaline cartilage regeneration in human trials. This scaffold is cellularized with autologous articular chondrocytes showing improved clinical scores in human trials [105].

8. Current clinical applications of multiphase designs

The restoration of osteochondral tissue damage should be focused on the physiological features and the structure of the tissues that make it up (cartilage and bone), considering the different microenvironments that coexist in the native tissue. Through tissue engineering, multiphase designs have been developed, such as those discussed throughout this chapter that aspire to achieve this goal. Currently, the vast majority of them have been characterized in vitro; some already have an in vivo analysis in medium and large species, which brings them closer to clinical application. Although there are few multiphase designs that are currently available for a clinical application, they open an important direction for the rigorous evaluation of the designs found on this path.

The Agili-C™ CartiHeal is a biphasic scaffold, which consists in of a cartilage phase made of hyaluronic acid and a bone layer comprised by crystalline aragonite (calcium carbonate based). After in vivo trial (goat model), this acellular scaffolds evidenced the potential to recruit cells from the host tissues, and enhanced hyaline cartilage formation and subchondral bone regeneration with

a continuous maturation process without deterioration of the repair tissue after 12 months implanted in critical osteochondral defects [111]. For clinical trials, only one clinical case has been reported in a 47-year-old man with an injury Outerbridge grade IV. The lesion was treated successfully and resumed normal activity after 18 months. In a follow-up at 24 months, restoration of the articular surface was demonstrated by MRI [112]. Although the results were encouraging, the occupation of the patient (athlete) could have a positive influence on the observed result, this makes it necessary to develop clinical trials in a larger number of patients under controlled conditions in order to extrapolate the benefits to a wider segment of the population.

The TruFit™ CB is an acellular scaffold made from polylactide-coglycolide copolymer and a bone phase containing calcium sulfate. The scaffold was used at first by direct implantation for the treatment of focal articular surface defects, but it showed some controversial results [113]. Several clinical studies have described a slow chondral restoration in the area of the lesion, due to poor bone repair [119], together with the poor integration of the implant with the surrounding tissue [120]. The long-term follow-up (up to 2 years) have also been controversial; however, the constant was delayed formation of the subchondral lamina [121]. Due to these clinical data, a thorough review of TruFit™ CB's design is necessary before arriving at an effective clinical application.

Maioregen™ is a triphasic biomimetic scaffold where the cartilage phase consists of equine type I collagen, an intermediate (tidemark like) layer consisting of type I collagen and magnesium-hydroxyapatite (Mg-HA), attached to the bone phase consisting of a mineralized blend of type I collagen and Mg-HA [116]. By preclinical tests on sheep and horses, it was possible to demonstrate the safety of the implant, but also that allowed the regeneration of the type II collagen-rich tissue after 6 months; this is a cell-free design [117, 118]. Throughout several clinical trials developed in such diverse populations ranging from 28 to 60 years and with a lesion size ranging from 1.5 to 6.0 cm^2, a good filling of the lesion and integration of the graft has been observed as a constant result. The evolution of the regeneration process has demonstrated the formation of subchondral bone and maturation of the chondral tissue in a period of 6 months. The evaluation by a high-resolution magnetic resonance imaging (MRI) shows the complete repair of the tissue in a period of 2 years in 66.7% of the cases treated, even in cases where the lesion involves the subchondral bone [116].

9. Conclusions

A cartilage repair treatment using tissue engineering comprises the implantation of bioabsorbable scaffolds that at first fill a chondral or osteochondral defect, then the production of cartilage repair tissue depends on the *de novo* synthesis of cartilage matrix elements. Such scaffolds support the local migration of cells (chondrogenic or osteogenic) that basically synthesize new extracellular matrix. The aim of all cartilage replacement strategies should focus on reconstruction of hyaline cartilage with its hierarchical organization; however, most of the current strategies based on monophasic designs lead to the production of fibrocartilage, which has inferior biological and mechanical characteristics compared to hyaline cartilage.

The design of multiphasic scaffolds aims at congruence with that of hierarchical nature, and from the studies that have been carried out over the past few years, it is clear that as a consequence, it substantially improves the integration of the implant with the surrounding osteochondral tissue, and positively influences the functional regeneration of both chondral and bone tissues. A vast array of multiphasic designs

has been evaluated in vitro; however, only three are currently available in the clinic; the question that arises is: how to optimize the efforts to achieve a conclusive clinical application?

The use of scaffolding in order to recapitulate as much as possible the hierarchical structure seems to be not enough. The decision to cellularize or maintain a cell-free scaffold is crucial, and the answer will depend on the 3D system in a particular way; therefore, cellularization in each of the chondral and bone phases must be taken into account for the final design. On the other hand, the inclusion of an in vitro maturing time of the cellularized implant is desirable; thus, at the time of implantation, the graft has enough mechanical characteristics to support the mechanical request in the joint.

The needed to mimic the ECM on a molecular level is another main goal that demands to be taken into account, so the bioactivation of the biomaterials with elements such as synthetic materials as the ceramics (tricalcium phosphate, hydroxyapatite, and bioactive glass), or even the same decellularized tissue matrix, turns out to be a valuable tool for cartilage design, since these materials enhance the growth of a bone-like layer to support the overlying cartilage to the existing osteochondral defect.

Experimental studies are ongoing to evaluate innovative multiphase designs regarding the interaction with cells and the environment in an *in vivo* framework. *In vivo* trials using small animal models provide innovative concepts in osteochondral tissue engineering; nonetheless, to reach the development of clinical trials in humans, it is important to follow successful experiments using animal models that have loads and joint dimensions similar to humans. Animals such as sheep, pigs, and horses have surgically created defect sizes ranging from 0.29 to 0.79 cm^2 and have average human-like defects depths of about 0.68–1 cm. The body weights of these animals are also comparable or much heavier than humans, which makes them more appropriate models to predict the results in clinical trials.

Although the challenge to incorporate the use of multiphase designs to the clinic is still great, from the results observed in the wide range of studies, it is possible to conclude that tissue engineering approaches based on multiphasic scaffolds represent a promising therapeutic treatment for the regeneration of osteochondral defects. Moreover, based on the clinical results, it seems that a three-phase approach offers the most promising results with patients.

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Conflict of interest

The authors declare that they have no conflict of interest

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