

Chapter 11

Osteochondral Tissue Engineering and Regenerative Strategies

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Abstract The orthopedic field has been facing challenging difficulties when it comes to regeneration of large and/or complex defects as we come across in osteochondral (OC) cases of lesions grade 4. Autologous OC mosaicplasty has proven to be a valid therapeutic option but donor site morbidity and the lack of long-term functionality remain sources of concern. OC tissue engineering has shown an increasing development to provide suitable strategies for the regeneration of damaged cartilage and underlying subchondral bone tissue. The use of two scaffolds with optimized properties for bone and cartilage architectures combined at the time of implantation as a multilayered structure was one of the first approaches for OC large defects regeneration. Last decade strategies using a bony-like scaffold supporting a cell layer for cartilage phase were introduced. Beyond the approaches already mentioned, three other strategies were reported for OCD regeneration. One methodology was the use of two different layers with a compact interface to create an integrated bilayered scaffold before cell seeding. A second strategy was the use of a single continuous structure but with different features in each layer. The last one was the combination of hydrogel phases creating this way the possibility to have injectable systems. These promising strategies for the regeneration of complex OCDs comprise the use of different biomaterials, growth factors, and cells alone or in combination, but the ideal solution is still to be found. The interface's mechanical properties have to be optimized. A different problem is related with the cell culture method within the 3D bilayered structures with heterogeneous properties. With the increasing demand of these stratified 3D structures new cell culture systems are required. Moreover these structures present the potential to be used as in vitro models, which is a need also because of the pressure resulting from the 3R's

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principle implementation that is now occurring. Regarding this, adapted bioreactors are being developed, but more efforts are required to target this scientific demand.

11.1 Introduction

Articular cartilage lines the end of our joint surfaces and is composed by chondrocytes inside a matrix made of collagen fibers, associated to glycosaminoglycan chains and elastin fibers [1]. In healthy joints, this unique and durable tissue allows bones to move against each other with minimal friction. Although articular cartilage comprises just one type of cells, chondrocytes become less active with age and injury [2]. Furthermore, the avascular nature of cartilage together with the declining function of chondrocytes with age contributes to the inability of full-thickness defects to heal spontaneously.

Articular cartilage damage arises as a consequence of both acute and repetitive trauma resulting in pain, effusion and/or mechanical symptoms, affecting directly individuals' life style [3]. Cartilage lesions in joints are characterized with different degrees. Superficial lesions, as fissures or cracks, are classified as grade 1. A grade 2 abnormality is defined when cartilage is affected up to 50 % of its thickness while grade 3 lesions are characterized by defects in which more than 50 % of the cartilage thickness, down to the subchondral bone but without bone penetration, is damaged. When areas of cartilage are worn away or torn away, exposing underlying subchondral bone, an OC defect (OCD), graded 4, is created [4]. If untreated, these lesions can progress to more-serious degenerative joint conditions, such as osteoarthritis [5].

Current clinical treatments for OCDs involve surgical approaches, such as microfracture, autologous and allogeneic cartilage tissue grafts, and autologous chondrocytes implantation (ACI) [6]. Among those, ACI using collagen membrane (AMIC) [6, 7] and matrix-assisted chondrocyte implantation, which are the first clinically approved TE approaches for OCD treatment, have been the most successful in achieving long-lasting cartilage repair. In fact these treatments are well established and effective to reduce patients' pain and to slow down the disease progression, however are not able to regenerate hyaline cartilage and completely restore patient's mobility. Therefore, the demand for new therapeutic options to regenerate OCDs is significant. Since the most promising results were obtained through regenerative medicine strategies, such as cell therapy or TE applying ACI and MACI, respectively, the development of a therapy for OC lesions treatment is nowadays focused on these fields.

TE concept is based on the combination of cells with materials. Structures are made of biodegradable and biomimetic materials to create networks with architectural and biochemical features similar to native extracellular matrix (ECM) [8]. In opposition to the chondrocytes used in ACI and MACI approaches, the exploitation of stem cells in combination with those 3D ECM-like structures presents a huge potential. These stem cells can be extracted from specific cells niches

and are promising for cell therapy and TE applications because of their high proliferative capacity and differentiation ability [9], as well as immunomodulatory role [10]. Moreover, improved in vitro 3D culture systems have been developed to maximize constructs features prior to implantation. The desired properties for a construct are being optimized to improve the ECM deposition and the mechanical performance.

This book chapter describes general regenerative medicine strategies mainly focusing in TE approaches for the treatment of OC lesions. The state-of-the-art of TE applied to complex OCD regeneration and the commonly used materials and their characteristics to create the support for host tissue invasion or transplanted cells growth is described and discussed. The strategies to recreate the bone and cartilage architectures as part of OC substitutes are disclosed and debated. Moreover, the sources of cells and their revealed efficacy for OC tissue regeneration will be analyzed. Finally the in vitro methodologies used to attain OC artificial constructs is scrutinized in terms of advantages and limitations.

11.2 Biomaterials-Based Strategies for OCD Regeneration

11.2.1 Strategies for OC Scaffolds Development

Biomaterials applied for bone and cartilage TE currently reported in literature present different physic-chemical characteristics for both parts. Most of the cartilage-like constructs are made of PGA meshes [11, 12], PCL prototyped structures [13], collagen [14, 15], hyaluronan [16, 17], chitosan [18] and gelatin porous sponges [19] and some approaches also applied external stimulus as, for example, a plasmid to induce TGF- β 1 transcription [20]. For bony scaffolds, ceramics, polymeric blends and composites are being used, such as blends of PLGA and PEG [21], TCP-reinforced PCL [22], bioactive glass [23], hydroxyapatite (HAp) with chitosan [24] or gelatin [20]. Additionally, as in cartilage, also in the bone part external factors are being applied to reinforce the OC differentiation as, for example, a plasmid encoding BMP-2 gene [20].

Several strategies are being considered for restoring the biological and mechanical OC functionalities. Specific biomaterial-based strategies are being proposed, including (I) different scaffolds for the bone and cartilage sides, (II) a scaffold for the bone component, but a scaffold-free approach for the cartilage side, (III) a bilayered scaffold with integrated interface, which can be a gradient or a compact layer, and, finally, (IV) injectable biomaterials as hydrogels (Table 11.1).

Several techniques and combinations of techniques have been applied with a direct correlation to create the scaffold with the desired characteristics for OC regeneration and the specificities of this complex interface. Different architectures have been reported; nanofibers based structures, which can be created by electro-spinning [25], sponges produced by freeze drying [24], or even agglomerated

Table 11.1 OC constructs design strategies for TE

Architecture	Advantages	Limitations	References
Two independent layers	Independent cell culture and differentiation	Poor integration between layers	[15, 28, 29]
Bone scaffold supporting cell free monolayer for cartilage	High control over cartilage layer phenotype	Poor integration and absence of transition zone	[30–32]
Bilayer structure with compact interface	Good integration between layers Control of cell migration Impaired vascularization in the cartilage part	Poor communication between subchondral bone and cartilage	[33–37]
Integrated bilayer structure	Good integration between layers Transition zone mimicking calcified cartilage part	Difficult cell culture regarding co-differentiation for osteo- and chondrogenesis	[14, 16, 18, 20, 27, 38–45]
Hydrogel based bilayered structures	Perfect fit and filling of defect High hydrated environment	Poor diffusion of nutrients in several hydrogel systems Poor cell anchorage in high hydrated environments	[46–50]

particles and microparticles [26] as 3D scaffolds. Porous scaffolds can also be created by salt-leaching technique [27], while well-organized matrices can be produced by rapid prototyping technologies [13].

11.2.2 Bone- and Cartilage-Like Tissues

Bone was one of the first tissues focused by TE, so it is also one of the topics with most developed state-of-the-art. Because of the chronologic development of the studies for bone TE, and of the markedly different tissue properties in OC interface, researchers started by independently addressing different scaffolds for bone and cartilage and then their integration as a single structure [28, 51, 52]. This integration has been achieved by sutures [29] and press-fit [30, 53], taking advantage of the natural body weight pressure, since the mechanical load can help achieving good contact in the interface sites and between neotissue and host tissue [54, 55]. Also, the cell culture period influences the bonding at the interface between cartilage and bone parts [54, 55].

Schaefer et al. [28] investigated the use of cartilage constructs using PGA meshes cultured with bovine calf articular chondrocytes. Bone constructs were created with a blend of PLGA and PEG cultured with bovine calf periosteal cells.

After independent culture (1 or 4 weeks), the cartilage- and bone-like constructs were sutured together. The resulting structure was cultured for additional 4 weeks which allowed attaining OC-like substitutes in vitro [28]. An in vivo trial would be interesting to test this approach.

In a different approach, Shao et al. [53] combined bone- and cartilage-like constructs by press-fit implantation. The scaffolds comprised PCL for the cartilage component and TCP-reinforced PCL for the bone component. After implantation in a load-bearing lapine model, the PCL/PCL-TCP scaffolds seeded with mesenchymal stem cells (MSCs) showed better results for OCD regeneration than the acellular control group. New bone formation was observed between 12 and 24 weeks, leading to the integration to host tissue. After 24 weeks of implantation, subchondral bone filled the scaffold and glycosaminoglycans and collagen type II deposition were observed in the cartilage region. However, new cartilage tissue lacked zonal organization [53].

The main disadvantage of this strategy is the poor interface between the two layers [56]. Moreover this strategy implies more extensive work in laboratory for the in vitro culture of separated bone- and cartilage-like parts before the combination of both parts, which raises further issues in the translation to clinics when scale up is required.

11.2.3 Bone-Like Tissue Plus Chondrocytes Sheets or Layers

Based on the outcomes of ACI and MACI approaches researchers have considered the creation of a 3D bone-like construct able to attach to the host tissue that is then topped with chondrocytes or a cartilaginous tissue layer. These are then expected to integrate the 3D bone-like structure reinforcing the stability of the cartilage-bone interface.

Using this strategy, most of the studies have taken advantage of ceramics as scaffold for bone regeneration [30, 57]. These were then combined with a cell sheet of chondrocytes or stem cells, as for example synovial stem cells [58], to stimulate the regeneration of the cartilage part [57, 59].

Acellular porous calcium polyphosphate (CPP) scaffolds were used as a substrate to grow articular cartilage on top. After implantation, the structures successfully supported loading up to 36 weeks, allowed bone ingrowth in the CPP substrate and were fixed by host native cartilage. However, some implants presented cartilaginous tissue delaminated between the 12 and 16 weeks period of implantation because of a low cartilage/CPP interfacial shear strength in comparison with the native OC interface [30]. Furthermore the use of ceramic scaffolds for the bone part raises mechanical limitation because of their hardness and lack of flexibility in comparison to some polymers. Consequently, this mismatch of

mechanical properties may lead to delamination between the bone scaffold and the new cartilage layer, which can be overcome by using polymer/ceramic composites.

Several biodegradable polymeric and composite materials were tested to overcome these mechanical problems, onto which neo-cartilage was produced in vitro. Porcine chondrocytes were seeded onto PLLA, PDLLA and collagen-HAP (Col-HA) scaffolds at high density in a closed and static bioreactor. PDLLA breakdown occurred in the first 11 days, leading to constructs of irregular shape and the highest amount of cell death. Low cell ingrowth and material breakdown was also evident in PLLA. Col-HA was the constructs formulation showing the superior results for OCD. These structures presented the highest cell viability and ingrowth and were also maintained with lowest degradation rate during the 15 days of cell culture, presenting the necessary integrity for further in vivo implantation after maturation. Furthermore Col-HA constructs also displayed collagen fibrils in neocartilage, contributing for the integration between cartilage- and subchondral bone-like tissue at the interface [32].

A different and important feature to reinforce the interface and to avoid the delamination problem is the calcified cartilage zone which was reported to be important for the interfacial shear properties [56]. In a study that anchored the cartilage tissue to CPP scaffold, the interface properties were enhanced as a result of the efficient integration of hyaline-like cartilage and the CPP phase by the calcified cartilage layer [60]. Although calcified cartilage could be formed by this strategy, the absence of a scaffold supporting the cell growth and ECM deposition of the cartilage layer can lead to a zonal organization failure of the neocartilage.

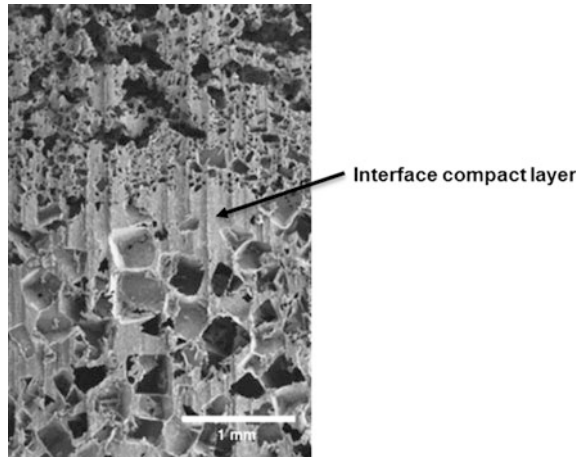
11.2.4 Bilayered Continuous Scaffolds-Based Strategies

There is also a category of bilayered scaffolds composed of two integrated layers for the cartilage and bone regions, or separated by a compact middle layer in the interface, to avoid mixing the two phenotypes. Like for the previously described approaches, ceramics and composites have been mainly used for the bone-like and natural or synthetic polymers for cartilage-like parts.

This strategy has been widely applied lately resulting in the development of several different structures for OC repair. A structure consisting of four layers, a porous CPP layer as bone component, a dense TCP layer to prevent blood vessel penetration, a porous CPP layer to fix bone and cartilage and a porous gelatin layer for the cartilage region [34].

To overcome the mechanical problems of ceramic-based scaffolds, a different study reported a structure composed of a composite and a blend of natural and synthetic polymers. The PLLA/HA or Bioglass[®] were used for the bone part and PLLA/starch blends for the cartilage part. This work considered that starch provides capability of water uptake and HA/Bioglass[®] to enhance bioactivity and thus HA formation on the bone side. The interface between cartilage and subchondral bone was integrated by a melt-based process (Fig. 11.1) [33].

Fig. 11.1 Bilayer structure presenting a compact interface layer obtained by melting. Reprinted with permission [33]. Copyright 2007, Elsevier



The compact interface presents the advantage of avoiding innervation and vascularization into the cartilage region. However this interface creates the disadvantage of interrupting the communication between both layers, impairing the paracrine effect from the MSCs of the subchondral bone.

An approach to solve some limitations associated to the poor invasion of host tissue into the bone-like part, combined a PGA woven for the cartilage part, a collagen I and HA coated porous PLLA/PCL foam for the bone part and a PLLA/PCL layer as the cartilage–bone interface. Vertical channels from the bottom layer to the upper border were created to allow the invasion of stem cells and blood from bone marrow after implanting following the mosaicplasty principle. The two phases of the scaffolds composed of PLLA and HA were assembled prior to cell seeding and implantation. These composite scaffolds were stabilized by using two bonded cylinders of PLLA and a thin PGA film was deposited between those two layers to prevent cell migration. These bilayers were co-cultured with osteoblasts and chondrocytes [35].

Despite this alternative thin but compact interface, the lack of communication between both new formed cartilage-like and bone-like tissue, which is important for good cell signaling, is also an issue. The poor connection present in most of the cases can compromise the natural integration of cartilage and subchondral bone through the calcified cartilage tidemark, which will not be created naturally.

A different strategy based on bilayered structures is based on the idea of having a homogeneous single integrated structure used for bone and cartilage regeneration, without a compact interface. The host tissue play the role of invading and defining the architecture between bone- and cartilage-like layers. Scaffolds incorporating or coated with various GFs, creating gradients have been proposed to stimuli simultaneous bone and cartilage regeneration [41]. Moreover, seeding with different cell lineages or stem cells stimulated with different GFs will promote the stratification inside the structure [61].

Bilayered scaffolds with integrated interface are usually formed by a composite and polymeric phase in a continuous structure for bone and cartilage layers, respectively. In contrast to a bony-like scaffold plus a cell-sheet for cartilage where neocartilage is generated from seeded chondrocytes on a subchondral support to form cartilaginous layer, bilayered scaffolds with integrated interface are designed to repair OCDs by using tailored bilayered structure, which mimic the structure of articular cartilage and subchondral bone tissue. The interface between engineered cartilage and subchondral bone parts is developed via fabrication methods including sintering [18], freeze-drying [14], salt leaching [27], emulsion [39], microspheres agglomeration [41], or even a CAD/CAM based process as the TheriForm™ [38, 62].

Oliveira et al. developed a HA/chitosan (HA/CS) bilayered scaffold by combining sintering and freeze drying techniques. The interface of the HA/CS bilayered scaffolds was achieved by partially impregnating the porous ceramic layer with the polymer one. Two distinct porous layers were obtained. Moreover, *in vitro* cell culture studies using MSCs demonstrated that both HA and CS layers provided an adequate 3D support for attachment, proliferation and differentiation of MSCs (Fig. 11.1) into osteoblasts and chondrocytes, respectively [18] (Fig. 11.2).

The first strategies proposing bilayered structures for OC regeneration are now being converted in gradient multiple layered structures to achieve a continuous layered structure. Levingstone et al. produced a layered construct by an “iterative layering” freeze-drying technique. The construct mimics the inherent gradient structure of healthy OC tissue: a bone layer composed of type I collagen and HAP, an intermediate layer composed of type I collagen, type II collagen and HAP, and a cartilaginous region composed of type I and type II collagen and hyaluronic acid (HA). This scaffold is currently being commercialized through the SurgaColl Technologies company, named ChondroColl™.

Recently, a multi-layered structure was developed by assembling a gelatin layer with layers containing different amounts of gelatin and HAP nanocrystals in which

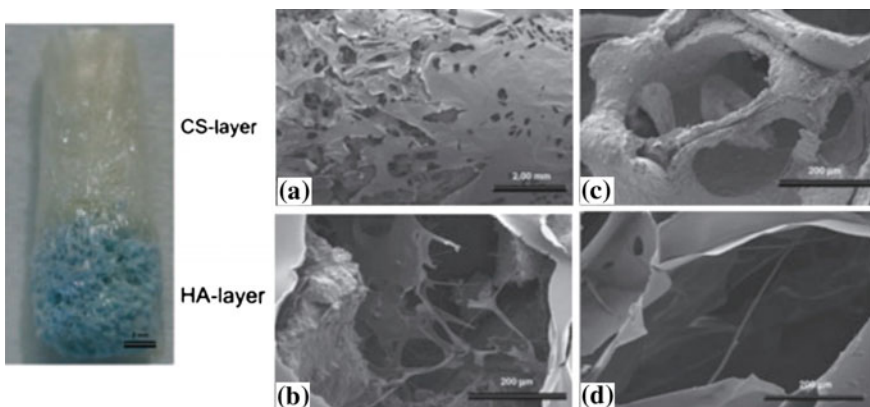


Fig. 11.2 Bilayered scaffold composed by a CS-based cartilage-like layer and a HA(sintered)/CS-based bone-like layer. Reprinted with permission [18]. Copyright 2006, Elsevier

a gelatin solution was used to stick the layers together. These scaffolds exhibit a high and interconnected porosity, and show mechanical properties that vary with the composition along the scaffolds. The *in vitro* co-culture of hMSCs results demonstrated that osteogenic and chondrogenic-differentiated hMSC influenced each other's behavior [45].

The bilayered structures seem to be promising for the regeneration of a tissue that presents two very distinct phases in terms of biology, but also in terms of architecture and physico-chemical properties. The presence of a hard interface can be an advantage to avoid the vascularization and innervation of cartilage tissue, however this hard interface avoids the crosstalk with the MSCs from subchondral bone. MSCs present a key paracrine effect over tissue regeneration, which seems to be more important than the anti-vascularization and -innervation role of the compact layer. Thus the continuous bilayered structure, presenting a gradient of structural and chemical characteristics from subchondral bone up to the top cartilage layer is a promising strategy for OCD regeneration.

11.2.5 Injectable Approaches

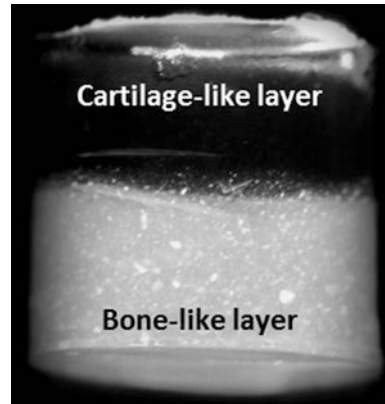
Commonly, biomaterials are used in OCD to create a 3D solid porous support for cell growth. Hydrogels were one of the last routes being explored in the field and are now produced using not only chemical crosslinking but also ionic [63] or photo-crosslinking [48, 64]. The use of these different types of crosslinking agents or starters fostered the development of injectable strategies for OCD regeneration because of the lower cytotoxic risk. The main advantage of this strategy relies in the ability of the hydrogel to occupy the defect shape. Moreover as part of a minimally invasive procedure, this is a more friendly method for a clinical approach.

Due to major concerns regarding the use of non-cytotoxic crosslinking agents and mild conditions, cells have been also easily mixed within the material before injection and polymerization, avoiding *in vitro* cell culture. By injecting a gel with encapsulated cells, an one-step procedure can be achieved introducing both components in a homogenized way [63]. The crosslinking can occur for example by ionic reaction with the host blood [65].

A robust integration of two different hydrogels without mixing both layers, designed for bone and cartilage has been difficult to obtain. Therefore, there are some studies combining hydrogels with hard scaffolds, such as composites or ceramics. Chondrocytes-containing agarose hydrogel was created for cartilage and osteoblasts-containing microspheres of PLGA and 45S5 Bioglass[®] for bone were fabricated in a cylindrical mould. An interface formed by chondrocytes embedded within a hybrid phase of gel and microspheres was achieved [46].

Recently, the proof of concept of a bilayer hydrogel integrating two different layers of low acyl gellan gum (LAGG) for cartilage part and LAGG with HAp particles for bone part, that can be ionically crosslinked *in vivo* was presented (Fig. 11.3) [63].

Fig. 11.3 Bilayered hydrogel composed of LAGG in the cartilage layer and LAGG incorporating HAp in the bone layer. Reprinted with permission [63]. Copyright 2014, Trans Tech Publications



The advantage of this last approach is mostly concerned with the defect shape, which can be completely fit by the injection of the hydrogels. The bone part can be easily adjusted in volume by eye, without requiring to previously knowing the exact volume of the defect, and then the cartilage part filled with the second layer of the hydrogel. Moreover this also allows encapsulating different cell types in the bone and cartilage layers at the time of implantation. The difficult host tissue invasion into the hydrogel, which can act as a barrier for tissue ingrowth and ECM deposition, can be seen as a main disadvantage of this approach.

11.3 Cells, Growth Factors and Gene Therapy for OCD Regeneration

Different cell sources have been used to obtain primary cells, as osteoblasts or chondrocytes, and stem cells for OCD regeneration. Osteoblasts and chondrocytes present the advantage of express the phenotype of the OC tissue, however low number of cells is obtained after isolation, requiring expansion *in vitro*, which is also very limited in the case of primary cells and usually leads to cell dedifferentiation [66].

Recently a new promising cell niche for articular cartilage regeneration was investigated by Pelttari et al. [67]. Adult human neuroectoderm-derived nasal chondrocytes, constitutively distinguished from mesoderm-derived articular chondrocytes by lack of expression of specific HOX genes, including HOXC4 and HOXD8, were shown, in contrast to articular chondrocytes, to be continuously reverted from differentiated to dedifferentiated states, conserving the ability to form cartilage tissue *in vitro* and *in vivo*. Moreover, those nasal chondrocytes are also reprogrammed to stably express HOX genes, typical of articular chondrocytes.

Stem cells can be isolated from several niches, as bone marrow, umbilical cord or abdominal fat. Recently, for OC TE, niches as fat pad, from Hoffa's body close

to the knee [68, 69], or synovial fluid [70, 71] have been tested. The limitation of low proliferation of primary cells and cell lineage dedifferentiation can be overcome using stem cells. Bone marrow, umbilical cord and abdominal fat are the common sources of stem cells, however new niches are being explored for OC application as mentioned above.

Scientists are now gaining interest in infrapatellar fat pad (Hoffa's body) to obtain MSCs for OC application, since the niche is present in the knee and the proximity to the lesion place plays an important role for the regeneration performed by cells [72]. Hoffa's body, which is a fat pad of adipose-derived stem cells (ASCs), has to be removed during an arthroscopy to facilitate the visualization of the knee and surgery handling, and also to avoid tissue inflammation as was explained before. This way, this tissue can also be considered as a promising source of ASCs with great potential to differentiate into chondrocytes and osteoblasts.

While primary cells represent native tissue phenotypes, stem cells have been either triggered and differentiated *in vitro* towards the lineages of interest or transplanted in an undifferentiated state. In this case a major concern related to *in situ* differentiation has supported the concomitant use of GFs [66, 73].

Designed structures for OCD regeneration are carrying GFs as TGF- β 1 for the chondrogenesis or BMPs for osteogenesis [74, 75]. Furthermore, structures presenting gradients of GFs are being explored. Microspheres are used to deliver the GF after implantation of the structures in the OCD [74]. For example, gelatine microparticles were used to carry IGF-1 and TGF- β 3 for cartilage phase. The results suggest that the dual delivery of TGF- β 3 and IGF-1, does not synergistically enhance the quality of engineered tissue [50].

GFs are also being used to promote selective cell differentiation and achieve the desired cell phenotype. However, with the more recently emerged gene therapy, this effect can be performed in a more constitutive and long term way [76]. Using a genetic modification strategy, chondrocytes overexpressing IGF-1 were cultured on biodegradable PGA scaffolds in dynamic flow rotating bioreactor up to 28 days. The resulting cartilaginous constructs implanted into OCD in rabbit knee joints lead to a spatially defined overexpression of IGF-1 enhancing articular cartilage repair and reducing osteoarthritic changes in the cartilage adjacent to the defect [77], which includes single parameters of cellularity, staining intensity and cluster formation [78]. Cellular morphology and architecture were significantly improved for defects receiving IGF-I constructs compared with those receiving lacZ constructs.

SOX trio is a genetic sequence of SOX-5, -6 and -9 very interesting for chondrogenesis and cartilage repair. Plasmid DNA (pDNA) containing the SOX trio genes was incorporated into a PLGA scaffold to slowly release, transfect ASCs and trigger chondrogenic differentiation. The *in vivo* study showed enhanced cartilage regeneration in ASCs/SOX trio pDNA-incorporated PLGA scaffolds [79]. In a different approach, SOX trio genes were also used for chondrogenesis, but in combination with Runt-related transcription factor 2 (RUNX2) for osteogenesis. A branched poly(ethylenimine) (bPEI)-HA delivery vector was loaded in a bilayered hydrogel mimicking native OC tissue. The spatially loaded combination of

RUNX2 and SOX trio DNA particularly in the bone part, significantly improved healing in relation to controls, hydrogels and factor alone [80].

11.4 3D In Vitro Cell Culture Methods

With the development of TE in the last decade, the need for 3D in vitro culture methods largely increased. The limited success obtained from the strategies tested until now to overcome disorders as osteoarthritis, for example, made the need to overcome their limitations using more realistic cell culture methods experiments. 3D cell culture, in vitro, present a huge potential to replicate in a better way several physiologic conditions that were not possible to correctly mimic in 2D or static cell culture. Furthermore, as a consequence of this huge introduction of new structures and methodologies to produce 3D scaffolds with different architectures, adaptable 3D in vitro culture systems have been developed as a need. There is a paradigm shifting occurring related with cell culture, which is changing from 2D to 3D, from static to dynamic conditions and from time-point analysis to real-time monitoring. The cell culture method is being adapted and bioreactors are emerging for TE field, which will be also useful to make the field of diseases in vitro modelling more realistic in the future.

A bioreactor can be described as a dynamic device or system for culturing cells or tissues under controlled conditions, either biochemical or mechanically. Several systems have been created; shake flasks (“mixed flasks”) [81] evolved to rotary vessels (“rotating vessels”) [82] and then to perfused chamber (“perfused cartridges”) [83]. Lately the incorporation of mechanical stimuli in the bioreactors has been followed in order to mimic the physical stress that occurs naturally in cellular environment [84, 85]. Generically in 3D cell culture, including the 3D structures for OC tissue engineering, cell sedimentation during the phase of cell adhesion is usually a problem, so the bioreactor should avoid this sedimentation [86]. Furthermore the cellular waste has to exit the interior of the structure being replaced by the fresh culture medium. Furthermore, bioreactors for OC TE have to be adapted for gradient or multi-layered structures and dual-environment cell culture conditions.

For OC TE and in vitro modelling, the mono-chamber bioreactors are evolving to dual-chamber bioreactors and compressive stimuli are also being included to create some dynamics mimicking the in vivo conditions. The first’s bioreactors used to mature chondrogenic and osteogenic constructs were not adapted for bilayered scaffolds, thus cells were cultured in separate environments using independent scaffolds for each part [85, 87, 88]. If the structure is multilayered, the culture chamber has to be adapted to offer the optimal culture environment to each layer giving rise to a different and specific engineered tissue [86].

In order to address the above mentioned need and despite the proposed above solutions, it is necessary to develop a reactor and a method to obtain engineered OC tissue grafts with 3D bi- or multilayered architecture that mimics the native tissue. Kuiper et al. designed a dual-chamber bioreactor for OC plugs and demonstrated

that the dual-chamber perfusion bioreactor positively influenced the co-culture of primary human chondrocytes and osteoblasts in the biphasic scaffold, in terms of cell viability, cell proliferation, ECM production and gene expression, however longer-term experiments have to be performed to evaluate the mechanical integrity of the cultured tissues [89].

For *in vitro* modelling of the osteoarthritic condition, a bioreactor presenting dual-chambers was designed for a high-throughput approach. This bioreactor system was fitted into a microfluidic device (Fig. 11.4). Each dual-chamber and insert of the 24 culture positions was fabricated using a stereolithography apparatus. The OC construct is supplied by two different culture mediums. The medium conduits are critical to create tissue-specific microenvironments in which chondral and osseous tissues develop and mature [90].

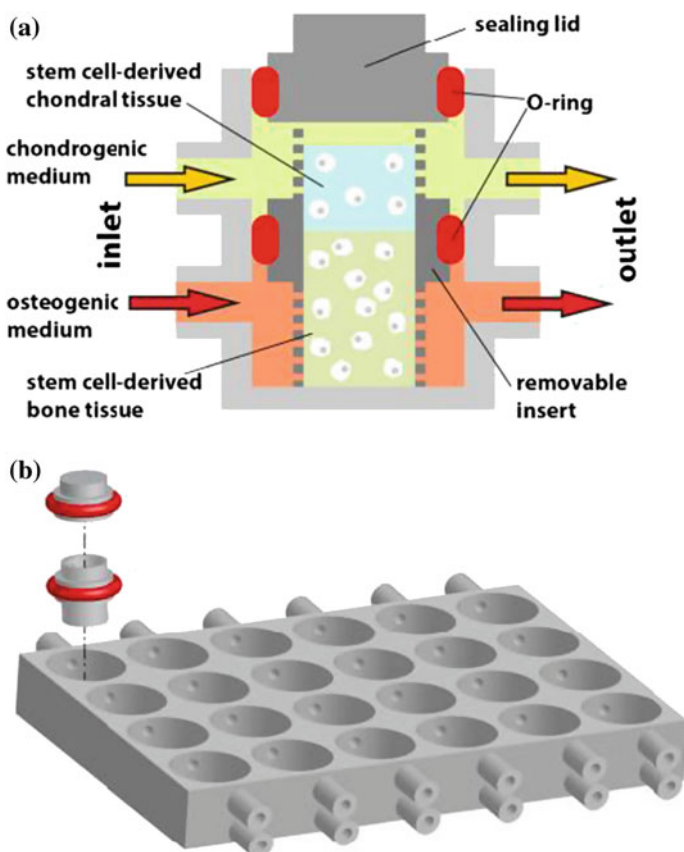


Fig. 11.4 Schematic of the bioreactor for OC *in vitro* model. **a** An individual bioreactor composed of the removable insert (*dark gray*) within a chamber (*light gray*) of the microfluidic plate (**b**) and fixed in place with two O-rings. Reprinted with permission [90]. Copyright 2013, American Chemical Society

In a different perspective, but also important in terms of physical stimulus, Nam et al. used a compressive system to test *in vitro* the expression of BMPs by osteoblasts and articular chondrocytes in 3D OC constructs culture under static and dynamic biomechanical stimulation (cyclic compressive strain). Biomechanical stimulation led to enhanced tissue morphogenesis possibly through this BMP regulation [91].

Combining the features of those systems reported above, a rotational dual-chamber bioreactor was patented describing the combination of a multi-chamber with physical compression and added also to the design the concept of rotational movements to improve cell distribution in 3D structures. This system can be used for OC TE flowing two different culture mediums and creating compression on top of cartilage-like layer [93].

Although some bioreactors are being developed for OC TE, the majority of them are being applied for constructs production. However bioreactors have the potential to be used as platforms for *in vitro* modelling of diseases as for example OA. These systems can contribute to recreate a dynamic and 3D environment which is more appropriate to mimic the natural OC tissue than a static and 2D cell culture of chondrocytes or osteoblasts.

11.5 Final Remarks and Future Directions

Despite the variety of materials, scaffold designs and cells that have been investigated for OC applications, an optimal strategy has not yet emerged. Therefore, more research efforts are needed to find suitable combinations of materials and methodologies that can be transferred to clinical practice.

Scaffolds for individual bone and cartilage tissue regeneration combined at the time of implantation represent one of the strategies for OC repair. These are based on the combination of different materials and cells specified for individual cartilage and subchondral bone tissues, or different biological factors capable to induce the selective differentiation of stem cells into chondrocytes and osteoblasts. The major limitation of this strategy is related with the lack of an interface, which contribute to the failure of the two layers under the stress created by the body weight, resulting in constructs delamination.

Another approach involves a scaffold for the bone component, but none for the cartilage component, have shown that bilayered scaffold-free cartilage constructs exhibit *in vitro* formation of cartilaginous-like tissue by chondrocytes seeded without the aid of biomaterial support. However, low interfacial shear strength at the interface between cartilage and the underlying bone scaffold is still a potentially vulnerable aspect of such systems. The formation of a mineralized layer in engineered cartilage has been suggested to solve this problem, considering that calcified cartilage is important for the integration of soft tissue (nonmineralized hyaline-like cartilage) and hard tissue (mineralized subchondral bone), and it can distribute the mechanical load across the interface. Although calcified cartilage could be formed

by this strategy, the generation of zonal organization in new articular cartilage might be inhibited by the lack of a cartilage-like scaffold for cell accommodation and tissue framework development.

Recently biphasic and multiphasic scaffold for OCD repair have been developed by TE. The biphasic scaffolds may present a compact interface. This compact interface can present the advantage of avoiding cartilage vascularization and innervation; however results in a non-naturally formed tidemark interface on the calcified cartilage transition zone. Moreover the communication between new cartilage and subchondral bone is important for the architecture, integration and maturation of the new formed cartilage. Following the progression of the bilayered structures, many researchers are now showing deeper interest in bilayered gradient scaffolds with continuous interfaces. With a graded scaffold, the interface would more closely resemble the native environment, allowing the interconnection of cartilage and subchondral bone, as occurs *in vivo*. Although there are some reported gradient biphasic scaffolds, it is extremely complicated to make a continuously gradient structure that allow smooth bone–cartilage interface. The combination of a continuous bilayered structure with a chemical ability to avoid vascularization and innervation in the cartilage layer can be a promising and key way to improve the current results using this strategy.

With the increasing understanding of the mechanical strengths, general structure, and the biology of bone and cartilage, the reconstruction of these two individual areas has led to improved OC constructs. Beyond the development of the scaffolds architecture, the use of GFs to form gradients and also the introduction of gene therapy for OC TE will potentially improve the quality of the engineered tissues.

Still, OC tissue repair needs the integration and interconnection between both tissues, which requires an advanced knowledge of how bone and cartilage interact. Understanding these two components separately allowed for the current state of the art. However, the true challenge in OC repair lies in the comprehension of the OC interface and its combined yet separate mechanical strengths, structure, and biology. This said OC TE tools and results improve with the understanding of OC interface architecture and phenotype. To further improve the mechanical strength, future studies were suggested to focus not only on using biochemical factors, but also mechanical stimuli.

The huge development of TE techniques and the now-how related with OC tissue can now be applied to the area of disease modeling. The *in vivo* (animal) models are nowadays the main models for drug effect screening before the clinical trials. However, there are efforts to decrease the use of animal models. The three Rs' (3Rs) principle, first described by Russell and Burch [92], is now being under higher attention to be applied for a better ethical use of animals in testing. This will create the need of new *in vitro* models to emerge in the drug development market to replace gradually the animal models to a certain extent. However, since the failure in predicting the efficacy or toxicity of a new drug carries huge costs for the industry, more reliable and realistic *in vitro* models are needed when compared to the existing ones, which are commonly 2D and static systems. This is a new born research field in OC related diseases.

More recently, iPS cells, which are generated by reprogramming somatic cells through the exogenous expression of transcription factors [93], improved the potential of autologous cell replacement therapies for regenerative medicine [94]. Nevertheless, and because of iPS technology is recent and is not completely controlled in terms of cell phenotype and in vivo functionality, no OCD regeneration studies are reported the use of this cells.

The huge variability of the OA tissues between individuals and the several joint tissues interplay are proven to be the most important challenges to be overcome. Thus, the development of a 3D OC model, with induced OA to provide the required reproducibility is expected to contribute to advances in the OA knowledge. In fact, we envision that the investigation of the disease in a controlled and reproducible way, will allow to identify new biomarkers for early OA diagnosis and to open up new possibilities for discovering new drugs for new therapies, with different efficiencies in the progressive stages of disease.

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