Composites Part B 259 (2023) 110736

Contents lists available at ScienceDirect

Composites Part B

journal homepage: www.elsevier.com/locate/compositesb

Bioreactor strategies for tissue-engineered osteochondral constructs: Advantages, present situations and future trends

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ARTICLE INFO

Handling Editor: Dr Hao Wang

Keywords:
Bioreactor strategies
Mechanical stimulation
Dynamic cultivations
Stress protocol
Tissue-engineered osteochondral construct

ABSTRACT

The aim of osteochondral tissue engineering is to achieve the complex, functional and three-dimensional tissue regeneration under well defined, controlled and reproducible conditions *in vitro*. To achieve tissue-engineered products *in vitro* that incorporate rapidly *in vivo* with healthy tissue, it is essential to develop high-performance cell/scaffold culture systems that mimic the dynamics of the *in vivo* environment. Bioreactors could provide specific physicochemical culture environment, suitable mechanical stimulation and controlled condition for the development of osteochondral constructs *in vitro*. This review highlighted the multifunction of bioreactor in tissue engineering, and presented microenvironment and biomechanics of native osteochondral tissue, to illustrate the necessity of establishing osteochondral constructs by bioreactor. Then, we especially emphasized the advantages and limitations of various bioreactors. Furthermore, we systematically summarized and discussed the development of bioreactor-based production systems for bone, cartilage and osteochondral tissue engineering in recent years. Finally, we made a simple conclusion and offered perspectives of bioreactor-based osteochondral tissue engineering. This review aims to serve as a reference for incorporating bioreactor strategies which could provide mechanical stimulation and physicochemical culture environment into the osteochondral construct culture regimens.

1. Introduction

Tissue engineering combines the knowledge of cells, engineering materials and biochemical factors for the development of biological substitutes that restore, maintain, or regenerate the damaged tissues to improve tissue function [1,2]. In general, autologous chondrogenic cells are seeded onto a biodegradable scaffold that supports their growth and chondrogenesis [3], then the cell-scaffold construct is cultivated with environmental factors appropriate for enhancing cell presentation. The aim of osteochondral tissue engineering is to mimic or imitate the biological osteochondral tissue to design and manufacture tissue-engineered substitutes which could provide an improved integration with the host tissue [4]. Many studies have focused on the development of highly biomimetic bone, cartilage, and osteochondral integrated biomimetic scaffolds [5–17]. However, promoting osteochondral defect repair is a very complicated task due to the different cartilage and subchondral bone composition along with their inherent biochemical, biological and biomechanical characteristics. Even with the perfect combination of growth factors, cells and scaffolds, osteochondral constructs still lack the physicochemical cues needed to successfully regenerate osteochondral tissue [18–20]. Moreover, a very important issue in tissue engineering is the formation of target tissues under dynamic cultivations, such as well defined, controlled and reproducible conditions. Compressive stiffness, toughness, strength, resilience and shock absorption are characteristics of joints, so depending on the site of implantation, some biomechanical requirements of osteochondral constructs must be fulfilled. Regarding cell-scaffold construct *in vitro* culture strategies, static culture methods often lead to gradual cell death and inactive ECM synthesis due to inadequate nutrient intake and waste transport, especially under

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https://doi.org/10.1016/j.compositesb.2023.110736
Received 16 February 2023; Received in revised form 30 March 2023; Accepted 13 April 2023
Available online 15 April 2023

long-term culture conditions. So, it is crucial to develop satisfactory strategies to provide suitable microenvironments and biological signals for the *in vitro* regeneration process of osteochondral tissue.

With the rapid development of stem cell-based therapies, people from both academia and industry started to realize that manufacturing a cell or tissue product needed industrial standardization. Moreover, the function of bioreactors in tissue engineering included cell seeding of porous scaffolds, nutrition of cells in the resulting constructs, and mechanical stimulation of the developing tissues [21]. Specifically, starting with a patient's tissue biopsy, the bioreactor system could isolate and expand cells, seed specific types of cells onto a scaffold, and culture the cell-scaffold construct until a properly developed graft is generated, thus performing different processing stages in a closed and automated system. In this process, environmental culture parameters and tissue development would be monitored and inputs fed into a microprocessor unit for analysis. The input data, combined with data from the patient's clinical records, automatically controlled culture parameters to predefined optimal levels and provide the surgical team with data on tissue development for timely implant planning (Fig. 1). Bioreactor systems could provide adequate nutrition and physicochemical stimulation to cells and/or cell/scaffold constructs, thus largely addressing some limitations of static culture methods. With a good bioreactor design, the culture conditions such as medium exchange and mechanical loading could be precisely and steadily controlled [22]. Particular focus of previous studies was given to the control over environmental conditions and to the automation of bioprocesses that bioreactors could offer. These features were essential not only for controlled fundamental studies of 3D tissue development but also to reduce manufacturing costs of engineered tissues and facilitate their broad clinical use. Bioreactors have been proven to be the key tools for initiating, maintaining and directing cell culture and tissue development in a three-dimensional, physicochemical defined, strictly controlled sterile environment. Together with biomechanical characterization, bioreactors could help in defining when engineered tissues have a sufficient mechanical integrity and biological responsiveness to be implanted.

Although bioreactor systems have been used in osteochondral tissue engineering, there are few reports about incorporating bioreactor strategies which could provide mechanical stimulation and physicochemical culture environment into the osteochondral construct culture regimens. This review aimed to provide a comprehensive overview of bioreactor-based osteochondral tissue engineering, including present situation and future perspectives. In order to illustrate the necessity of using bioreactors to establish osteochondral constructs, this review began with the microenvironment and biomechanics of native osteochondral tissue at the tissue and cell-level, followed by the multifunction of bioreactor. Then, we especially emphasized the advantages and limitations of various bioreactors for tissue engineering. Moreover, we summarized and discussed the development of bioreactor-based production systems for osteochondral tissue engineering. Finally, we made a simple conclusion and offered perspectives of bioreactor-based osteochondral tissue engineering.

2. Microenvironment and biomechanics of osteochondral unit

It has been known that cells and tissues react to external mechanical stimuli that may include gravitational and hydrostatic pressures, and shear stresses caused by fluid flow. To some extent, the quality of cartilage and bone-like tissue generated *in vitro* is currently restricted by a limited understanding of the role of biomechanical stimulation and physicochemical culture parameters in regulating tissue development [23,24]. Therefore, in order to develop tissue-engineered products *in vitro*, it is essential to understand the microenvironment and biomechanics of native osteochondral tissue and/or cells and tissues reaction to external mechanical stimuli.

2.1. Osteochondral tissue

According to the cellular (phenotype and morphology), extracellular matrix (ECM) (composition and structure), and biochemical (growth factors and cytokines) gradients along the depth of the tissue, the load-bearing osteochondral tissues composed of cartilage, calcified cartilage, and subchondral bone at the ends of long bones in the joints [25–27]

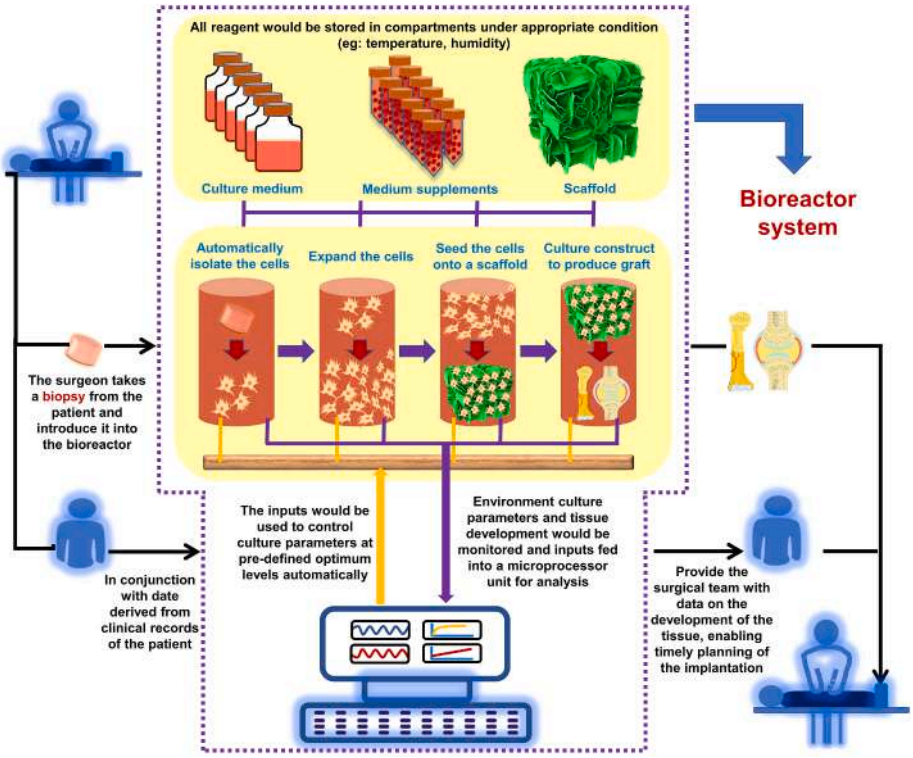

Fig. 1. Schematic diagram of tissue-engineered grafts produced automatically by a closed bioreactor system.

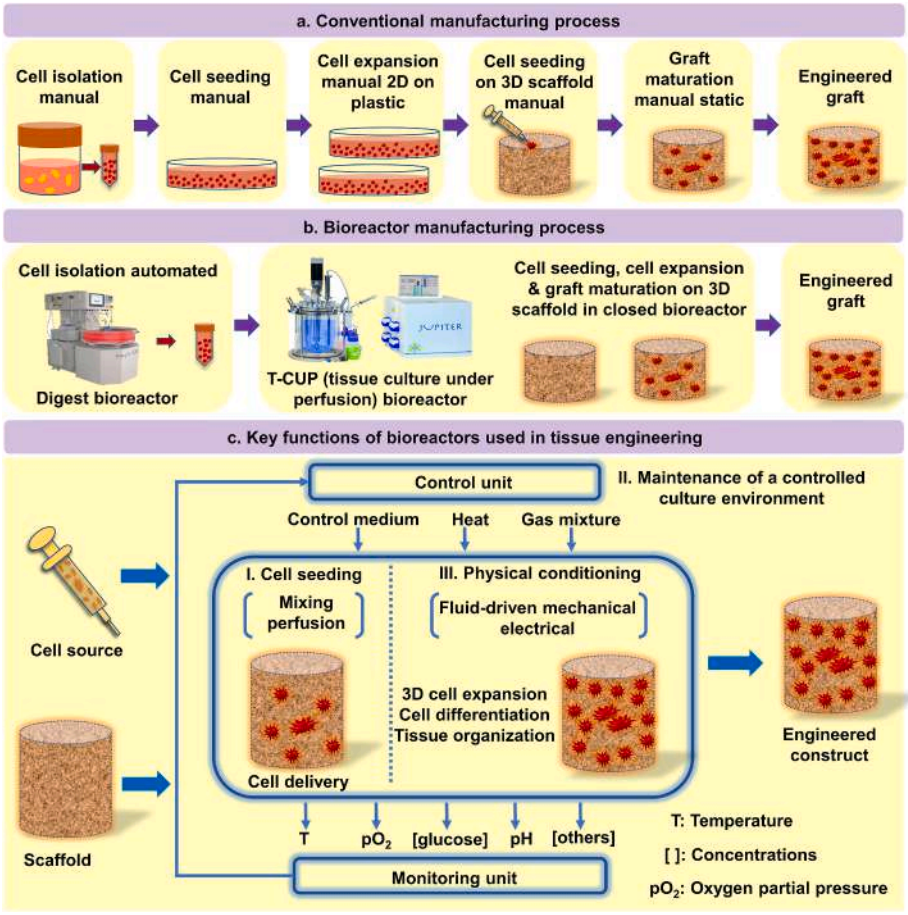

Fig. 2. The schematic diagram of microenvironment and biomechanics of osteochondral unit: a) The schematic diagram shows the tissue-level microenvironment and biomechanics of osteochondral unit, including force distribution and intensity along the osteochondral depth upon application of compressive and shear (red arrows); b) Cell-level biomechanical studies: the single chondrocyte approach to elucidate mechano-transduction pathways and to select biomechanical forces as exogenous stimuli for tissue-engineered strategies. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 2a). Hyaline cartilage is a stratified and multilayered tissue, including superficial zone, transitional zone and deep zone [28]. The elastic modulus of subchondral bone is approximately 3 orders of magnitude greater than that of cartilage, while that of calcified cartilage is an intermediate value [29]. Such a naturally varying elasticity in this interfacial tissue enables cartilage to significantly minimize bone-bone impact loading while maintaining structural integrity of the joint. When the structural gradient is disrupted, the tissue loses its ability to absorb mechanical loading, ultimately resulting in joint failure [30].

2.1.1. *Hyaline cartilage zone*

Articular cartilage is the avascular connective tissue that covers the ends of bones in diarthrodial joints and provides a nearly frictionless bearing surface. Articular cartilage in adults is a comparatively acellular tissue, and occupied by an extensive extracellular matrix that is synthesized by chondrocytes. 70–80% of the matrix wet weight is water, while the remaining solid fraction is primarily collagen type II (50–75%) and proteoglycans (15–30%) [31]. At the macro-scale, adult hyaline cartilage is a multi-zonal material which divided into three layers representing different ECM compositions, orientations and cell phenotypes. The superficial layer is covered by a thin acellular layer (lamina splendens), usually a few hundred nanometers, which serves as a low-friction surface [32,33]. This combination of molecules confers the superficial zone tissue with the highest permeability and an optimized capability to withstand shear forces from the articulating surfaces [34–36]. In addition, this zone is important for the compressive strength of the cartilage tissue and possibly in isolation of cartilage from the immune system [36,37]. As shown in Fig. 2a I, proteoglycan is a family of compound whose basic structure may be represented by a single protein ‘core’ to which glycosaminoglycan (GAGs) chains are covalently attached. The major GAGs in articular cartilage are chondroitin sulphate, and smaller amounts of keratan sulphate also exist. Due to a higher concentration of GAGs, the permeability of the transitional zone matrix is also lower than that of the superficial zone and supports moderate compressive forces [34]. The deep zone has the largest diameter collagen fibrils, the lowest cell density, the most proteoglycan, the maximum concentration of GAGs and the least water compared to the superficial and transitional zones [38]. This combination of molecules imparts the deep zone tissue with the lowest permeability, with practically no fluid flow permitted through the tissue, and an optimized capability to resist the highest interfacial shear forces.

Native articular cartilage endures thousands of sustaining compressive loading cycles per day, and major weight-bearing joints are subjected to compressive stress ranging from 0.5 to 7.7 MPa, typically resulting in about 13% strain [39]. The compressive aggregate modulus of joint articular cartilage ranges from 0.08 to 2 MPa, and varies along the depth of the tissue [40]. A creep indentation studies of cartilage indicated that the patellar groove exhibited the lowest Poisson’s ratio, lowest aggregate modulus and highest permeability. When negatively charged proteoglycans trap fluid within the cartilage matrix during joint loading, native articular cartilage encounters 3–10 MPa hydrostatic pressure physiologically [41,42]. Hydrostatic pressure can minimize damage to the ECM during *in-vitro* stimulation as hydrostatic pressure does not shear or deform the essentially incompressible tissues. Collagen in the cartilage matrix imposes tension that allows the tissue to swelling without rupturing to maintain a constant static pre-tension of the natural articular cartilage [39].

2.1.2. *Calcified cartilage zone*

The fibers in deep zone extend into the tidemark, a basophilic line of

unknown composition, that indicates the beginning of calcified cartilage tissue. The calcified cartilage layer shows gradual thinning with the increase of age during tissue reconstruction, along with tidemark duplication in subjects over 70 years old [43]. Mature articular cartilage is integrated with subchondral bone through a 20–250 μm thick layer of calcified cartilage. Articular calcified cartilage is a mineralized layer within which hypertrophic chondrocytes are embedded in a mineralized matrix of types II and X collagen, as well as proteoglycans. In biology, osteochondral interface serves as a barrier to inhibit vascular invasion from bone layer and prevent the mineralization of hyaline cartilage layer. Such a barrier is essential for maintaining the integrity of repaired cartilage over time. In mechanics, this zone bears significant shear stress due to the interface between the soft cartilage and much stiffer bone, which provides cushioning mechanical property support for the upper and lower layers.

2.1.3. Subchondral bone zone

Subchondral bone zone is a nano-composite material mainly composed of glycoproteins, such as collagen, laminin and fibronectin, and nano-sized hydroxyapatite (HA) [44]. HA crystalline plate-shaped particles with a length of 20–80 nm, a width of 15 nm, and a thickness of 2–5 nm is deposited on type I collagen fibrils, which provides the tissue's stiffness and compressive strength [45,46]. The compressive modulus of subchondral bone (5.7 GPa) is higher than that of cartilage. Bone is an extremely complex and highly vascularized tissue with a unique capacity to heal and remodel, which could provide many essential functions, such as structural support for the body and serve as a mineral reservoir, withstands load bearing and protects internal organs [47]. The subchondral zone be divided into cortical bone (compact bone) and cancellous bone (trabecular bone) according to the distribution of blood vessels and porosity [48]. Cortical bone is a dense connective tissue with few blood vessels and characterized by cylindrical elements named osteons (also known as Haversian systems) surrounded on both sides by inner and outer circumferential lamellae (regular layers or sheets of tissue) [49]. Cortical bone represents 80% of the skeletal mass and therefore supports most of the mechanical function. Cancellous bone shows a spongy-like structure that is highly vascularized, and contains lamellae interconnect each other in order to form a trabecular tissue mesh. Trabecular bone is approximately 80% porous, which is filled with marrow that involved in generation of red and white blood cells [45]. Bone is the complex and multilayered tissue that exhibit anisotropy [50], tension-compression asymmetry [51], and deformation rate effects [52,53].

2.2. Biomechanics of single cell

In addition to tissue-level biomechanical studies of osteochondral tissue, mechanics have been investigated at the cellular level. Physiological mechanical stimulation to promote the synthesis of chondrocyte ECM protein is crucial for maintaining the function and integrity of articular cartilage [54]. Quantifying single cell mechanics by modeling the biological responses to cellular deformation has elucidated the chondrocyte responses to loading and mechano-transduction at the single cell level (Fig. 2b), which could inform the selection of appropriate stimuli for tissue engineering. Substrate-dependent adhesive forces exhibited when articular chondrocytes were cultured on different substrates. By quantifying the force required for cell detachment from a substrate, we could indirectly determine the ability of different substrates to support cell adhesion. The effect of shear stress upregulated chondrocyte proliferation and induced an increase of protein synthesis in the superficial region of articular cartilage. Statically compressing chondrocytes could modulate gene expression of ECM proteins in a dose-dependent manner [55,56], and increased force exposure catabolically shifted single cell mRNA levels of aggrecan, collagen type II, and tissue inhibitor of metalloproteinase-1. The compressing chondrocytes could stimulate cartilage specific gene expression which related to ECM synthesis and maintenance. Fig. 2b II is a schematic diagram of the microstructure of osteoarthritis.

3. The role of bioreactors in tissue engineering

Conventional manufacturing processes and bioreactor-based production systems used to produce the engineered grafts are shown in Fig. 3a and b. Conventional manufacturing, based on traditional benchtop manual culture methods, are lengthy, labor-intensive and would possess inherent variability among operators, which poses challenges towards regulatory compliance and cost-effective production. As an alternative, automated bioreactor-based manufacturing systems have the potential to overcome these limitations associated with conventional manufacturing methods, such as eliminate the requirement for large and expensive Good Manufacturing Practice (GMP) tissue engineering facilities and minimize operator handling. Moreover, by automating and standardizing the manufacturing process in controlled closed systems, bioreactors could save production costs and thus facilitate engineered osteochondral grafts clinical acquisition at a larger scale.

Bioreactor is not only a powerful technical tool to support and direct the development of living and functional tissues in a three-dimensional *in vitro*, but also a dynamic culture model system to study the basic mechanisms of cell function under physiologically relevant conditions. In particular, the roles and functions of state-of-the-art bioreactor equipment are as follows (Fig. 3c): 1) Dynamically seeding cells in a three-dimensional matrix: bioreactors can maximize the cell utilization, control the cell distribution and improve the reproducibility of the cell seeding process; 2) Overcome the diffusion limitation of traditional static culture environment: bioreactors that monitor and control culture parameters can provide well-defined model systems to investigate fundamental aspects of cell function and can be used to enhance the reproducibility and overall quality of engineered tissues [57]; 3) Bioreactors do not only increase mass transport inside three-dimensional structures but also reduce the handling steps, hence reducing contamination potential; 4) Physically stimulating the developing constructs: bioreactors that apply physiological regimes of physical stimulation can improve the structural and functional properties of engineered tissues; 5) Serving as valuable *in vitro* models to predict the responses of an engineered tissue to physiological forces on surgical implantation, and to study the pathophysiological effects of physical forces on developing tissues; and 6) By quantitative analysis and computational modeling of stresses and strains of normal tissues *in vivo* and engineered tissues in bioreactors, bioreactors could thus help to define when engineered tissues have a sufficient mechanical integrity and biological responsiveness to be implanted [58], to determine potential regimes of physical rehabilitation that are most appropriate for the patient receiving the tissue [59].

4. Bioreactors types and stress protocols used for osteochondral tissue engineering

In order to develop osteochondral tissue engineered products *in vitro*, it is essential to develop adequate cell/scaffold culture systems that mimic the dynamics of the *in vivo* environment. Up to now different bioreactor designs have been proposed to dynamically culture cells in osteochondral constructs, including spinner flasks, rotating-wall bioreactors (that simulate the effect of microgravity), perfusion-based bioreactors, compression bioreactor and double-chamber stirred bioreactor (Fig. 4a). Different stress protocols were applicable to bioreactor-based osteochondral tissue engineering (Fig. 4b), including basic mechanical and/or hydrostatic or hydrodynamic forces when using a bioreactor. Basic forces could be applied in static (gravity), hydrostatic (continuous fluid pressure) and mechanostatic systems (continuous pressure over a lever). Dynamic forces were applied via fluid flow, shear and an electric field or motor-driven devices. Each bioreactor model had its own advantages and limitations, either in terms of system complexity

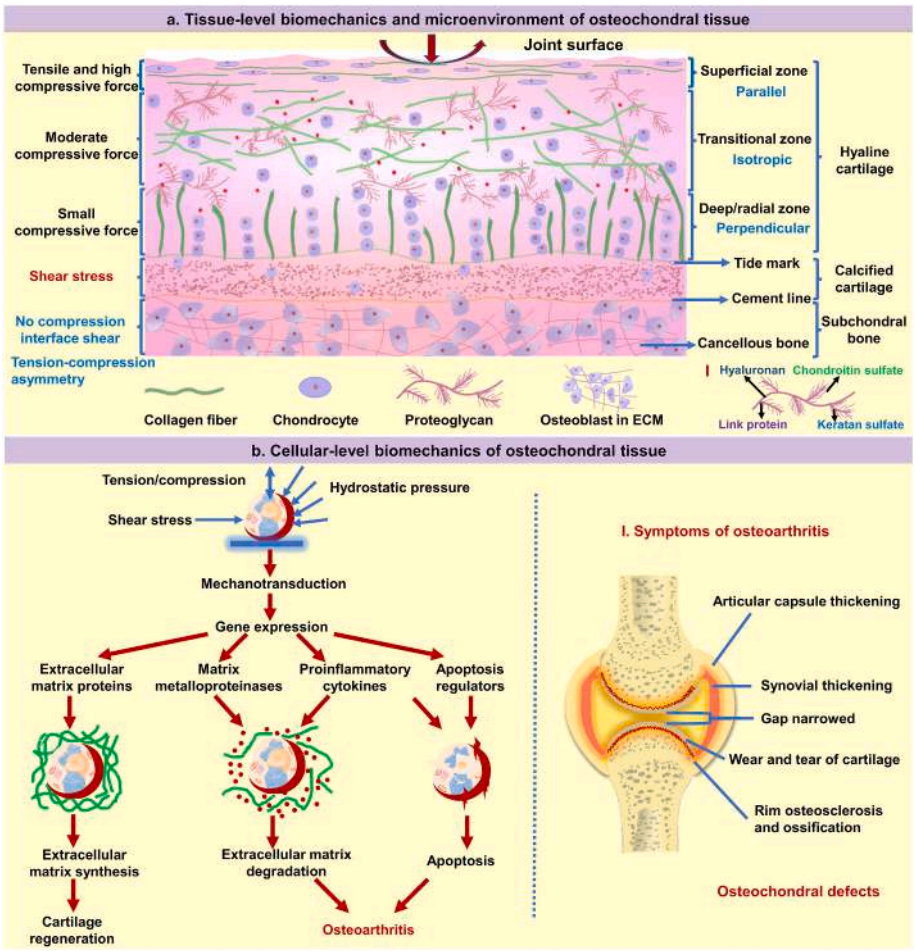

Fig. 3. Schematic representation of conventional manufacturing process (a) and bioreactor-based production systems (b) used to produce the engineered grafts; (c) The key functions of bioreactors used in research applications for tissue engineering: (I) Cell seeding of three-dimensional matrices; (II) Maintenance of a controlled culture environment; (III) Physical conditioning of cell/scaffold constructs.

and operations or in terms of cell/construct culture. Mechanical loads acting on tissue-engineered osteochondral constructs during mechanical stimulation were illustrated in Fig. 4c, including direct compression, shear stress, hydrostatic pressure, biaxial tension and uniaxial tension. It is well known that these physical stimuli could modulate cartilage and bone metabolism and modulate the ECM production when applied in specific mixtures with specific intensity and frequency. In this section, the different kinds of bioreactors were discussed, and the advantages and disadvantages of them were summarized.

4.1. Spiner-flask bioreactor

The spinner flask is one of the simplest bioreactor designs, as shown in Fig. 4a I. Specifically, scaffolds seeded with specific cells by convection were attached to needles hanging from the lid of the flask. Medium was added into flask to cover the cell-scaffold construct and changed every few days to ensure high nutrient concentrations. During the whole culture process, the medium in flask was stirred continuously with a magnetic stirring bar at the bottom of the flask to generate convective forces allowing continuous mixing of the media surrounding the scaffolds, which induced mixing of oxygen and nutrients throughout the medium and reduced the concentration boundary layer at the construct surface [60]. Inevitably, mechanical mixing also generated turbulent eddies or induced unwanted shear gradients in the reactor vessel, which were detrimental to tissue development.

4.2. Rotating-wall bioreactors

A rotating-wall bioreactor, originally designed to simulate the effect of microgravity, could maintain cells in microgravity state and present low fluid shear stress [60,61]. The most common rotating-wall bioreactor was the slow turning lateral vessel consisting of two concentric cylinders (Fig. 4a II): the stationary inner cylinder had a membrane that allows gas exchange, while an outer cylinder made of impermeable material was primarily responsible for rotation, and the space between the two cylinders contained the freely suspended tissue-engineered constructs and was perfused continuously with media [62]. A rotating-wall bioreactor could be used for both microcarriers and scaffolds in a microgravity state without considering mechanical mixing, which could avoid cell deposition and promote cellular interactions [61]. Specifically, pre-implanted cell/scaffold constructs or chondrocytes and microcarriers were placed inside the bioreactor, then the cylindrical vessel rotated horizontally around its axis, which maintained a constant state of free-fall (simulating microgravity conditions and inducing dynamic laminar flow conditions) by adjusting the rotation speed of the outer cylinder so that the centrifugal force just balanced the gravity and the fluid resistance on the internal object [63]. In this environment, the movement of the scaffold/microcarrier relative to the media resulted from the small amount of inevitable settling [61,65]. The rotating-wall bioreactor was an efficient suspension culture vessel to produce dynamic laminar flow, reduce diffusional limitations of nutrients and wastes, and minimize the turbulence and shear stress in culture compared to the spinner flask model [64,65].

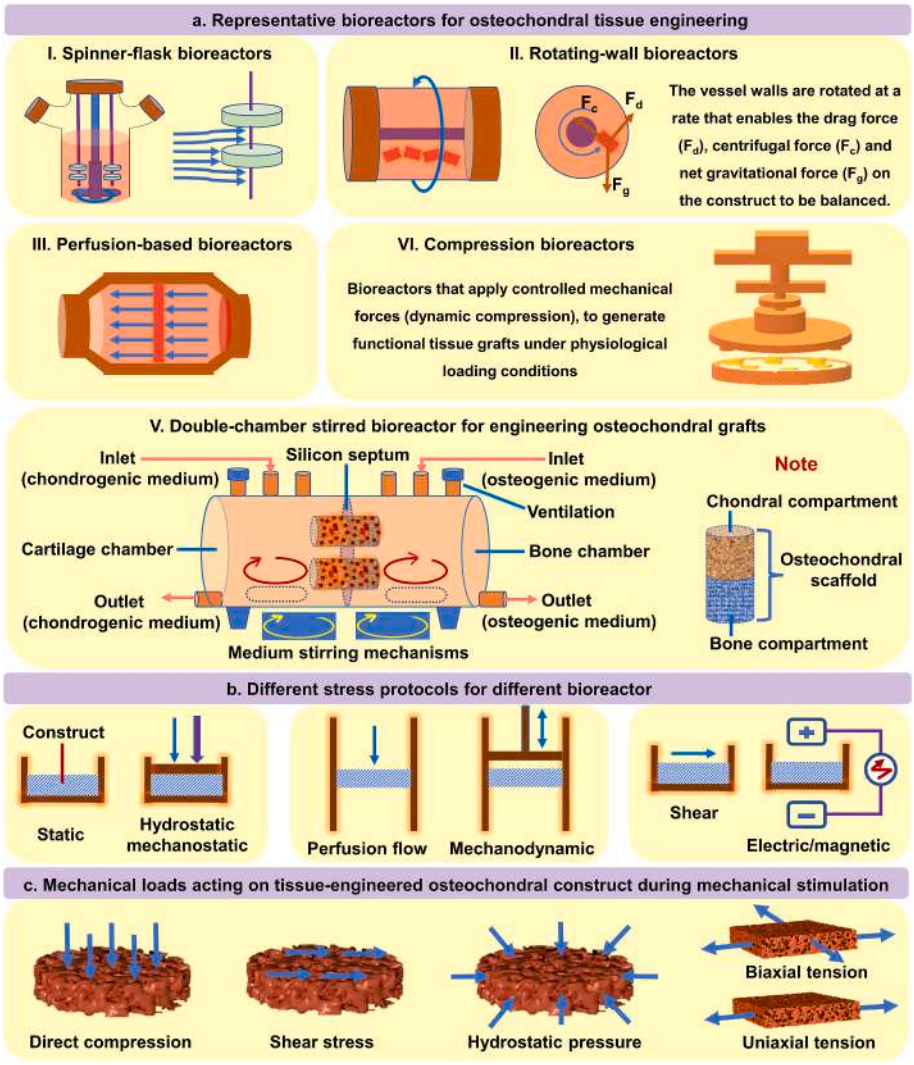

Fig. 4. Bioreactor strategies used for tissue-engineered osteochondral constructs: (a) Bioreactor types including spinner-flask bioreactor, rotating-wall bioreactor, perfusion-based bioreactor, controlled mechanical bioreactor and double-chamber stirred bioreactor; (b) Schematic overview of different stress protocols applicable to scaffold constructs; (c) Schematic representation of mechanical loads acting on tissue-engineered osteochondral constructs during mechanical stimulation, including direct compression, shear stress, hydrostatic pressure, biaxial tension and uniaxial tension.

In particular, three-dimensional cell culture in a rotating-wall bioreactor was used to identify and control biochemical factors that affected cell function, tissue growth and integration capacity [66]. Microcarrier suspensions could be used to culture individual cells in a bioreactor rather than seeding cells in a scaffold, which could enhance the maintenance of chondrocyte differentiation phenotypes. Microcarrier beads of various types, such as collagen or dextran, were suspended in a reactor with a continuously stirred medium and chondrocyte inoculum was added. The media was exchanged every few days throughout the culture process. Cell doubling times were around 2–3 days in these bioreactors versus 5 days in Petri dishes, which demonstrated that the increased mass transfer in the bioreactor promoted cell growth.

4.3. *Perfusion-based bioreactor*

Perfusion culture system could be used for seeding and/or culturing three-dimensional constructs, in which mass transfer was improved and mixing was achieved by fluid recirculation rather than impeller motion (Fig. 4a III). During seeding and/or culture, scaffold could be placed in fluidized beds or airlift reactors in a press-fit manner so that the medium was forced to pass through the center of the samples and hooked to a perfusion system, and then perfuse media flowed through tissue-engineered constructs in a controllable and continuous manner by a peristaltic pump, yielding a highly uniform cell distribution and high seeding efficiency throughout the full engineered scaffolds [64,67]. In the case of perfusion-based bioreactors, culture media could flow through the interconnected pores of a tissue-engineered construct, which enhanced mass transfer not only at the periphery but also within its internal pores. In addition, perfusion-based bioreactor improved environmental control and physical stimulation of the cells in large constructs, thus overcoming the difficulties of simpler models [68].

Direct perfusion of cell suspensions through 3D scaffolds has proven to be an effective method to enhance cell distribution throughout scaffolds and contribute to the formation of more homogeneously distributed tissues [69]. Previous studies have indicated that perfusion culture enhances cell survival, growth and function: compared to the static control group, the higher cellularity was observed after 9 days of perfusion culture [70]; when the perfusion culture lasted for more days, the tissue have histological and mechanical characteristics similar to natural tissue. Moreover, direct perfusion seeding methods overcomed the operator-dependent limitations associated with cell static loading, such as micro-pipetting [71]. However, the effect of direct perfusion may be highly dependent on the medium flow rate and the maturation stage of the construct [72]. Therefore, to optimize the perfusion-based bioreactor for tissue engineering, it is essential to achieve a careful balance between the fluid-induced shear stresses within the scaffold pores, the mass transfer of nutrients and waste products to and from cells, and the retention of newly synthesized extracellular matrix components within the construct. For example, these systems could be

integrated into a more complex bioreactor setup, where cells were first perfused through a scaffold and then maintained in a specific dynamic culture within an engineered structure. Although direct perfusion bioreactors have been shown to promote osteocyte growth, differentiation and mineralized matrix deposition [73,74], as well as GAGs synthesis and accumulation in chondrocytes [72,75], it is difficult to culture different phases of osteochondral construct simultaneously due to variations in inflow rate and culture time [72,76,77].

4.4. Compression bioreactors

Increasing evidence suggests that mechanical conditioning might increase the biosynthetic activity of cells, improve the structural and functional properties of engineered tissues, and thus possibly accelerate tissue regeneration *in vitro* [59]. In this context, the controlled mechanical bioreactors must be combined with quantitative analysis and computational modeling of the physical forces experienced by tissue engineered constructs, which could provide the controlled environments for reproducible and accurate application of specific mechanisms of mechanical forces to three-dimension constructs [65]. Compression bioreactors were designed to mimic the *in vitro* physiological environment of bone and were characterized by the requirement for repeated mechanical stimulation to achieve functional bone regeneration. As shown in Fig. 4a VI, the compressive force was transferred to the construct through flat plates that distribute the load evenly, ensuring that the construct receives uniform stimulation [78]. Generally, compression bioreactor systems consist of a motor, a system that provides linear motion, a controlling mechanism that provides displacement regimes, and a compression chamber that applies static or dynamic compressive loads directly to the cell/scaffold constructs.

4.5. Double-chamber stirred bioreactors

The double-chamber bioreactor with two tubular-shape glass chambers was separated by a silicone-rubber septum with multiple holes to hold the biphasic scaffold, forming two independent medium-circulation systems. As shown in Fig. 4a V, each of chamber of double-chamber bioreactor has four branch tubes for medium inflow and outflow, oxygen ventilation, and surplus use. Magnetic-bar stirring provided mixing of the medium and mechanical stimulation for osteochondral constructs. The whole double-chamber bioreactor could be autoclaved as an aseptic ventilation filter is connected to one branch tube for each chamber under laminar flow. The bioreactor contained two independent media recirculation and stirring systems, and thus could be used to support coculture of different kinds of cells or induce mesenchymal stem cells to differentiate into chondrocytes and osteoblasts in the same biphasic scaffold. For engineering osteochondral grafts, osteochondral constructs traversed to a silicone membrane that separated the double-chamber stirred bioreactor into two chambers, so that the cartilage and bone compartments of the osteochondral constructs could be cultured independently within its respective chamber. Limited diffusion of the media may occur inside the scaffold, which may facilitate micro-interactions between different cells at the interface, forming osteochondral interface.

Although compression and hydrostatic pressure bioreactors offer many advantages, it is still necessary to further understand its specific mechanical loading and application mechanisms, including intensity, frequency, continuous or intermittent use, and application duration [79]. Additionally, complex systems including dynamic compression and moderate perfusion have been developed to provide adequate stimulation to osteochondral engineered constructs. As the engineered tissues require different mechanical conditioning at different stages of maturation, the double-chamber stirred bioreactors with spatial and time-dependent loading stimulation have been used to ensure the formation of fully functional neo-tissue that can be integrated into the host [21]. It should also be noted that the complexity of these bioreactor systems for large-scale production and/or high-throughput applications has prevented their widespread use. Therefore, considering the complex requirements of osteochondral tissue engineering, current studies have begun to explore the combination of different types of bioreactors to better simulate the osteochondral physiological environment *in vitro* [80–83].

5. Development of bioreactor-based production systems for osteochondral tissue engineering

Bioreactor design for osteochondral construct culture was based on parameters or forces affecting chondrogenesis, osteogenesis and osteochondral interface formation. Bioreactors assisted in replicating critical site-specific properties of host tissue by creating uniform cell concentrations within biological scaffolders, and providing key physical and chemical cues for tissue regeneration. Previous studies have reported that dynamic laminar flow patterns accompanied by compression could enhance cartilage ECM stimulation, and medium perfusion could influence the geometry, distribution, and orientation of bone-like trabeculae of bone and provide shear stress to stimulate neo-bone formation [84,85]. If the two layers of the biphasic scaffold were cultured separately (Fig. 5 Strategy A), its versatility may be increased. If the integrated bi-layered scaffolds were to be cultured simultaneously, then new strategies should be adopted where typically a double chamber was required to differentiate the culture conditions. Different strategies were designed to produce well-integrated bi-layered osteochondral constructs prior to double-chamber stirred bioreactor, such as two different cells or cells from common progenitor cells were seeded in the two layers of the scaffold (Fig. 5 Strategy B). Table 1 summarized the development of bioreactor-based osteochondral tissue engineering.

5.1. Bioreactor application in two independent layers of osteochondral construct (strategy A)

For osteochondral tissue engineering, its versatility may be increased if the two layers of the biphasic scaffold were cultured separately. Bioreactor studies showed that mechanical compression and shear were two main physical forces affecting cartilage and bone phase [86]. Currently, direct compression by hydrostatic pressure has applied to stimulate adult human mesenchymal stem cells [87–89]. Fluid shear was the use of fluid to generate shear forces between osteochondral constructs to increase the transfer of wastes and nutrients during culture. In this section, we presented and discussed the application of bioreactor configuration with different load conditions in bone and cartilage tissue engineering.

5.1.1. Bioreactor-based bone layer construct

As we all known that the bone was a highly structured, mechanically active three-dimensional tissue, the true biological environment of osteoblasts stemmed from dynamic interactions between active cells subjected to mechanical forces and the ever-changing three-dimensional matrix architecture. Therefore, mechano-stimulation was an important input in maintaining and/or inducing the bone phenotype [90,91]. In particular, compression loading acted as a key physical cue in natural bone, significantly increasing osteogenic markers [92].

Goldstein et al. [74] examined three different culturing schemes (a spinner flask, a rotary vessel and a perfusion flow system) to test their ability to promote cell growth and osteogenic function in porous Poly (DL-lactic-*co*-glycolic acid) (PLGA) foams. PLGA foam discs were seeded with osteoblastic marrow stromal cells and cultured in the presence of dexamethasone and L-ascorbic acid for 7 and 14 days. Although these culturing schemes yielded similar cell densities, the higher ALP activity and better cell uniformity throughout the cultured foams was observed in a rotary vessel or in a flow perfusion system, which demonstrated that culturing techniques that utilize fluid flow, and in particular the flow perfusion system, improve the properties of the seeded cells over those

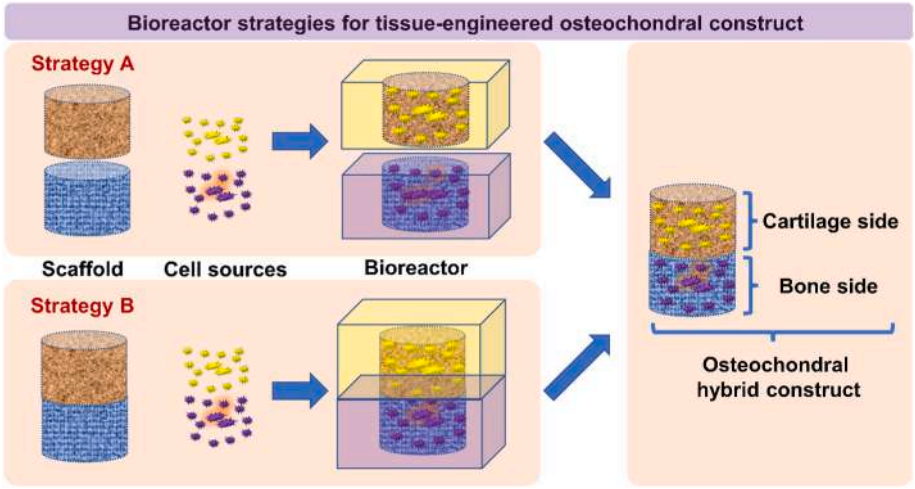

Fig. 5. Two possible bioreactor-based strategies for osteochondral tissue engineering. Strategy A: the cell culturing is performed independently in two sides, which are integrated before implantation; Strategy B: two different cell sources or common progenitor cells are seeded in the two sides of a bi-phasic scaffold that contains different differentiating agents, and then cultivated in a special bioreactor with two separated chambers.

maintained in static culture. Zhang et al. [57] systematically compared biaxial rotating (BXR) bioreactor with three most commonly used bioreactor systems: spinner flask, rotating wall vessel and perfusion bioreactors, for their application in bone tissue engineering (Fig. 6). Design and working patterns of bioreactor systems was shown in Fig. 6a. All the bioreactors with human fetal mesenchymal stem cell (hfMSC) cultured on scaffolds maintained a high cellular viability throughout the 4 weeks, while cellular scaffolds under BXR and Perfusion bioreactor culture showed more cellularity than the other two bioreactors culture (Fig. 6b). Quantitative analysis showed a significantly higher neo-tissue volume and more uniform spatial distribution of neo-tissue in BXR bioreactor group compared to others (Fig. 6c). As shown in Fig. 6 d, quantitative ALP activity assay showed the highest level of ALP activity in BXR bioreactor cultured scaffolds than the others at week 2 and 4. Analysis of calcium deposition in the scaffolds revealed significantly higher calcium deposition in BXR bioreactor cultured scaffolds than the others. This study indicated that BXR bioreactors were superior to other bioreactors in hfMSC proliferation, spatial distribution and osteogenic induction. Mitra et al. [93] highlighted the need to optimize perfusion culture duration of tissue-engineered constructs to achieve the desired level of bone formation. To systematically investigate the effects of static and dynamic approaches on cell seeding and *in vitro* culturing of a scaffold-containing perfusion bioreactor system, Beşkardeş et al. [94] seeded MC3T3-E1 pre-osteoblasts on chitosan-hydroxyapatite scaffolds under different flow rates and directions. The results proved that static seeding combined with perfusion culture enhanced cell viability and osteogenic differentiation.

In 2019, Gandhi et al. [95] investigated the effect of a tubular perfusion bioreactor system for the growth and differentiation of bone mesenchymal stem cells (BMSCs) seeded onto fibrin. Experiment result showed that MSCs cultured in the tubular perfusion bioreactor system resulted in increased vascularization and mineralized tissue formation *in vivo* relative to static culture. To induce the osteogenic commitment of BMSCs seeded on of 3D chitosan-graphene templates, Lovecchio et al. [80] developed a standalone perfusion/compression bioreactor system specifically (Fig. 7a), allowing the administration of perfusion flow through a dedicated peristaltic pump and application of a compressive axial deformation through a custom-made mechanical loading platform driven by a precise stepper motor, and including an automatic media replacement system to minimize the cell culture contamination risk, as illustrated in Fig. 7b. After using the dynamic culture protocol, considerable increase in cell number within the inner core of the scaffold and enhanced extracellular matrix mineralization was observed, compared to traditional static culture conditions (Fig. 7d). These observations showed that novel standalone perfusion/compression bioreactor system would be suitable for investigating the osteogenic phenotype commitment of stem cells. To determine whether osteogenic preconditioning could improve the bone-forming potential of unfractionated bone marrow aspirate (BMA), Harvestine et al. [96] perfused cells on ECM-coated scaffolds to generate naïve and preconditioned constructs, respectively. The results demonstrated that bioreactor-based preconditioning augmented the bone-forming potential of BMA. Based on these studies, it is possible to design more complex systems for osteochondral constructs, including bioreactors that simulate multiaxial joint motion [97] or perfusion seeding systems [98]. Interestingly, Fragomeni et al. [99] established a three-dimensional model of transient tracer transport based on the radial-flow packed-bed bioreactors, which may overcome the transport limitations of static and axial perfusion bioreactors in the long-bone tissue engineering. In 2020, Nokhbatolfoghahaei et al. [100] studied the effect of various bioreactor designs (including rotating & perfusion and perfusion bioreactors) on extracellular matrix synthesis. Osteogenic assessment of scaffolds revealed that rotating & perfusion bioreactor led to significantly higher expression of OCN and RUNX2 genes and also greater amount of ALP and collagen I protein production compared to static groups and perfusion bioreactor. Therefore, multi-stimuli seem to be more effective in bone engineering. In 2021, to investigate the mineralization of a titanium wire mesh scaffold under both static and dynamic culturing, Dua et al. [101] constructed and validated an *ex-vivo* bone bioreactor culture system that could maintain the viability of bone samples for an extended period *ex-vivo*. The results demonstrated that a bone bioreactor could be used as an alternate tool for *in-vivo* bone ingrowth studies of new implant surfaces or coatings. Schädli et al. [102] presented an *in vitro* dynamic compression bioreactor approach to monitor bone formation in scaffolds under cyclic loading. After seeding with BMSCs, time-lapsed imaging of scaffolds in bioreactors revealed increased bone formation in hydroxyapatite scaffolds under cyclic loading, and immunohistological staining further confirmed this stimulating effect. Suzuki et al. [103] constructed a tissue-engineered bone by culturing rat bone marrow cells (RBMCs) onto porous apatite-fiber scaffolds using a radial-flow bioreactor. The results showed that the ALP activity and osteocalcin content of calcified cells tended to increase with the culture period, and the differentiation of tissue-engineered bone could be controlled by varying the culture period.

Table 1
The development of bioreactor-based osteochondral tissue engineering.

Construct composition	Bioreactor type	Main findings	Ref.
Bone tissue engineering			
Osteoblastic marrow stromal cells seeded PLGA foam	Spinner flask, Rotary vessel and Perfusion flow bioreactors	Culturing techniques that utilize fluid flow, and in particular the flow perfusion system, improve the properties of the seeded cells over those maintained in static culture	[74]
hfMSC-mediated macroporous polycaprolactone/ tri-calcium phosphate scaffolds	Biaxial rotating bioreactor, Spinner flask, Rotating wall vessel and Perfusion bioreactors	This study indicated that BXR bioreactors are superior to other bioreactors in human fetal mesenchymal stem cell (hfMSC) proliferation, spatial distribution and osteogenic induction.	[57]
Chitosan-hydroxyapatite scaffolds	Perfusion bioreactor Stress: shear stresses	Perfusion seeding followed by static culture neither increased the mitochondrial activity nor enhanced the expression of osteogenic genes of cells except for osteopontin gene. However, static seeding combined with perfusion culture enhanced cell viability and osteogenic differentiation	[94]
Mesenchymal stem cells encapsulated in fibrin beads	Tubular perfusion bioreactor Stress: shear stresses	Mesenchymal stem cells cultured in the tubular perfusion bioreactor system resulted in increased vascularization and mineralized tissue formation *in vivo* relative to static culture	[95]
hBMSC-seeded 3D chitosan-graphene templates	Standalone perfusion and compression bioreactor Stress: low-shear stress and higher-compressive stresses	After using the dynamic culture protocol, there was evidence of a larger number of viable cells within the inner core of the scaffold and of enhanced extracellular matrix mineralization, compared to traditional static culture conditions	[80]
Mesenchymal stem cells seeded gelatin/ β-Tricalcium phosphate scaffold	Rotating & perfusion bioreactor VS perfusion bioreactor	Osteogenic assessment of scaffolds after 24 days revealed that rotating & perfusion bioreactor led to significantly higher gene expression and greater amount of ALP and collagen I protein production compared to static groups and perfusion bioreactor. The outcomes demonstrated that rotating & perfusion bioreactor action on bone regeneration is much preferable than perfusion bioreactor.	[100]
Mineralized titanium wire mesh scaffold	*Ex vivo* bioreactor Stress: shear stress and compressive stresses	This study demonstrated that *ex-vivo* bone bioreactor is capable of keeping the bone alive in an *ex-vivo* environment for extended periods of up to 7 weeks, which is sufficient time for bone formation in animal models	[101]
A biopolymer incorporate hydroxyapatite or a mixture with barium titanate nanoparticles	Dynamic compression bioreactor Stress: compressive stresses	By combining mechanical loading and time-lapsed imaging, this *in vitro* bioreactor strategy may potentially accelerate development of engineered bone scaffolds and reduce the use of animals for experimentation	[102]
Rat bone marrow cells (RBMCs) seeded porous apatite-fiber scaffolds	Radial-flow bioreactor Stress: shear stress	The employment of radial-flow bioreactor and apatite-fiber scaffolds provided a favorable 3D environment for cell growth and differentiation, which provided valuable insights into the design of tissue-engineered bone for clinical applications	[103]
Cartilage tissue engineering			
Meniscus cell-seeded PLLA constructs	Spinner flask bioreactor Stress: hydrostatic pressure	Growth factors and hydrostatic pressure can be used successfully in combination to enhance the functional properties of *in vitro* engineered knee meniscus constructs	[109]
Agarose gel	*In vivo* bioreactor (subperiosteal space)	Using an agarose gel biomaterial, large volumes of bone can be engineered de novo within the *in vivo* bioreactor without cell implantation and administration of growth factors	[111]
Goat bone marrow cells (GBMCs) seeded starch–polycaprolactone fiber mesh scaffolds	Flow perfusion bioreactor Stress: shear stresses	The enhanced mRNA expression for chondrogenic genes and ECM production indicated that flow perfusion system can create a favorable environment to promote the chondrogenic differentiation of GBMCs cultured onto SPCL fiber meshes	[70]
3D cell culture construct	Perfusion microcell culture system Stress: dynamic compressive loading	The metabolic activity of chondrocytes was significantly affected by the stimulating frequency at the higher compressive strain of 40% (2 Hz, 40% strain)	[112]
fibrin-polyurethane scaffold	Cartilage bioreactor mimicking the complex motion of an articulating joint *in vivo* Stress: dynamic compressive and shear loading	Multidirectional loading, consisting of axial compression and ball oscillation, promote maintenance of chondrocyte phenotypes through up-regulation of cartilage gene expression	[97]
Self-assembled neocartilage	Tunable device using an orbital shaker to create oscillatory fluid motion with fluid-induced shear stress Stress: fluid-induced shear stress	An optimal range of fluid-induced shear (FIS) stress, 0.05–0.21 Pa, improved the compressive modulus of neocartilage *in vitro*. Implanted neocartilage treated with a combination of FIS stress and bioactive factors remodeled *in vivo*, yielding a 122% increase in collagen content and 168% increase in Young's modulus	[114]
Osteochondral tissue engineering			
Bone: Sinbone block (calcium-phosphate block) + porcine chondrocytes Cartilage: Gelatin + porcine chondrocytes	Double-chamber bioreactor	After 4 weeks of culture, the chondrocytes retained their phenotype as proven immunohistochemically, and hyaline-like cartilage with lacuna formation could be clearly seen in the gelatin scaffold on the surface of the calcium phosphate. The results of *in vitro* study show that this biphasic scaffold can support cartilage formation on a calcium-phosphate surface in a double-chamber bioreactor	[115]
Bone: Chitosan/hydroxyapatite (HA) scaffolds Cartilage: Chitosan scaffolds	Double-chamber bioreactor	Bi-layered chitosan-based scaffolds are developed based on the optimization of both polymeric and composite scaffolds by particle aggregation, which overcome any risk of delamination of both polymeric and composite parts designed, respectively, for chondrogenic and osteogenic components that are mechanically stable	[116]

(*continued on next page*)

Table 1 (*continued*)

Construct composition	Bioreactor type	Main findings	Ref.
Bone: hBMSCs + 10% Methacrylated Gelatin (mGL)/1% hydroxyapatite/0.15% LAP (w/v) HBSS solution Cartilage: 10% mGL/1% Hyaluronic Acid (mHA)/0.15% LAP (w/v) HBSS	Microfluidic-based multi-chamber bioreactor	The tissue specific gene expression and matrix production as well as a basophilic interface indicated that a new microfluidic-based multi-chamber bioreactor can facilitate osteochondral differentiation and toxicity testing	[117]
Bone: Mineralized blend of type I collagen and Mg-HA + ovine chondrocytes Cartilage: Equine type I collagen + ovine chondrocytes	Cartilage digestion bioreactor and T-CUP perfusion bioreactor	By demonstrating the safety and efficacy of bioreactor-generated grafts in two large animal models, results suggested that bioreactor-generated grafts accelerate the repair of acute osteochondral defects, compared to cell-free scaffold implants, and may result in a more robust repair in the longer term	[118]
Photocrosslinked gelatin scaffolds + human mesenchymal progenitor cells (iMPCs)	Dual-flow perfusion bioreactor	After 28 days of culture, a micro-physiological biphasic osteochondral tissue chips with cartilage in the top and bone in the bottom were successfully formed, and chondral and osseous phenotypes were validated by specific gene expression and matrix deposition	[119]
Cell seeded cylindrical fibers	Hollow-fiber bioreactor	Exosomes produced from 3D culture of U-MSCs in a hollow-fiber bioreactor increased the number and enhance the function of chondrocytes by stimulating cell proliferation, migration, and matrix synthesis, and inhibiting apoptosis	[113]

5.1.2. *Bioreactor-based cartilage layer construct*

Articular cartilage is a mechanically-sensitive tissue that could respond to biomechanical stimuli. This section describes bioreactors with biomechanical stimuli for stimulating cartilage explants or engineered constructs, including direct compression, hydrostatic pressure, shear bioreactors (surface/fluid-surface shearing, direct flow and fluid perfusion, low-shear "microgravity" bioreactors), and hybrid bioreactors incorporating multiple loading regimes [104]. The points of contact between the femoral condyle and the tibial plateau cause compression within the cartilage tissue, which is a major component of normal mechanical stimulation within diarthrodial joints. Previous studies demonstrated that under the correct conditions, bioreactors that provided direct compression could stimulate chondrocytes, which could increase the synthesis of proteoglycan and collagen and improve mechanical properties [105]. Moreover, the low shear system could stimulate cells in the matrix while still allowing the cells to express their chondrocyte phenotype [105]. Recently, researchers have begun to combine various bioreactor culture systems with external mechanical stimulation (such as hydrostatic pressure, direct compression, and shear stress) to improve the quality of cartilage construct [106–108].

Gunja et al. [109] investigated the combinatorial effects of TGF-β1 and hydrostatic pressure on meniscus cell-seeded PLLA constructs. The

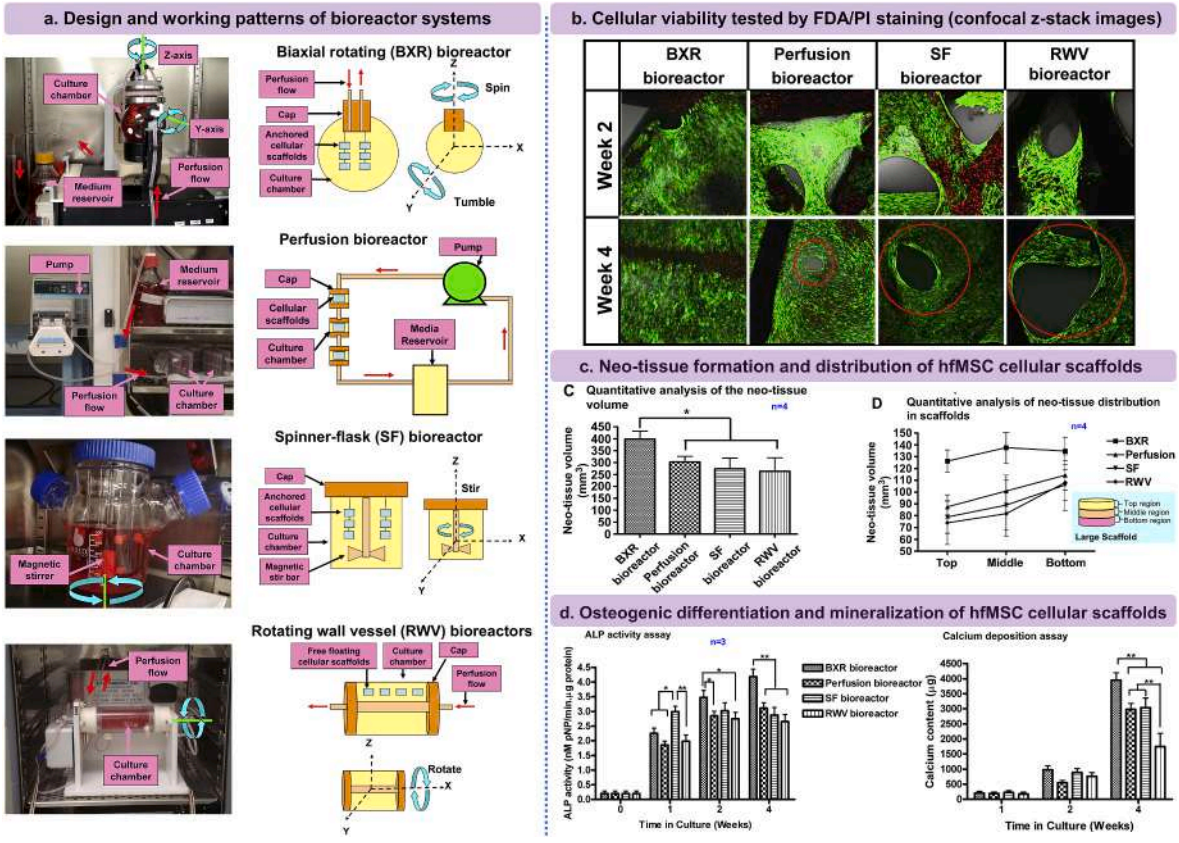

Fig. 6. Systematically compared biaxial rotating (BXR) bioreactor with three most commonly used bioreactor systems: spinner flask (SF), rotating wall vessel (RWV) and perfusion bioreactors, for their application in bone tissue engineering: a) Design and working patterns of bioreactor systems; b) Cellular viability tested by FDA/PI staining (confocal z-stack images); c) Neo-tissue formation and distribution of hfMSC cellular scaffolds; d) Osteogenic differentiation and mineralization of hfMSC cellular scaffolds [57]: copyright 2010, Elsevier.

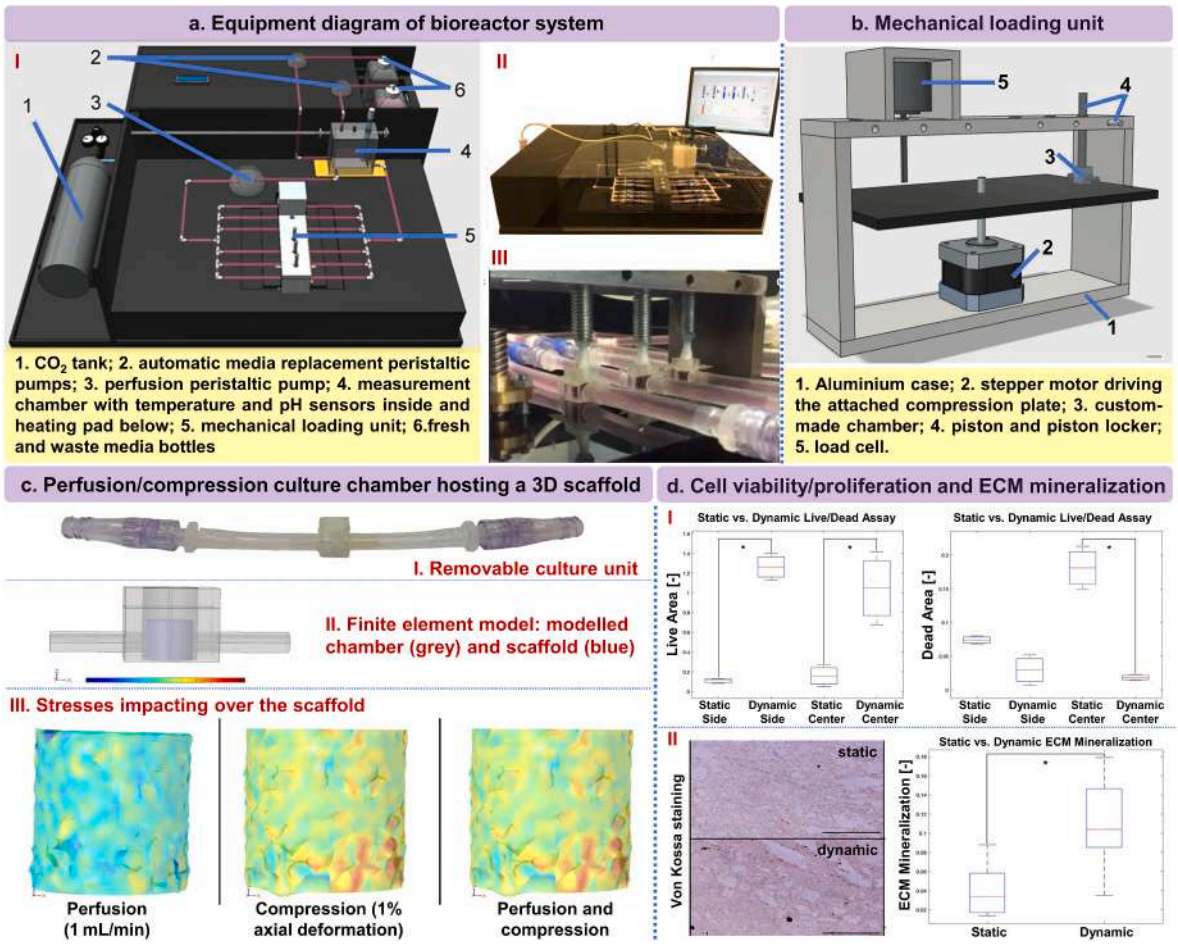

Fig. 7. A standalone perfusion/compression bioreactor system was proposed to address the osteogenic commitment of stem cells seeded on of 3D chitosan-graphene templates: a) Equipment diagram: (I) front view, (II) Actual bioreactor prototype, (III) Detail of the cell culture perfusion/compression unit; b) Mechanical loading unit that apply mechanical compression onto seeded scaffolds hosted within the custom-made chambers; c) Perfusion/compression culture chamber hosting a 3D scaffold: In stresses impacting over the scaffold, the predominance of a light blue color shows low-shear stress, the yellow/orange color distribution shows higher-compressive stresses; d) Cell viability/proliferation (I) within the 3D scaffold under either static or dynamic (perfusion and compression) culture conditions; (II) Optical microscopy images of ECM mineralization (Von Kossa staining) within the core of the 3D scaffold, and quantitative analysis of the total amount of scaffold area covered by the von Kossa. Reproduced with permission [80]: copyright 2019, Springer. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

higher collagen deposition, higher GAG deposition and enhanced compressive properties indicated that growth factors and hydrostatic pressure can be used successfully in combination to enhance the functional properties of *in vitro* engineered knee meniscus constructs. Fan et al. [110] demonstrated that the intermittent static biaxial tensile strains could improve chondrocyte metabolism and tissue engineered cartilage constructs growth. In addition, treatment of full-thickness damage to hyaline cartilage was hampered by the limited availability of autologous healthy cartilage and the lengthy, cost-prohibitive cell isolation and expansion steps associated with autologous cartilage implantation (ACI). To address these problems, Emans et al. [111] proposed a strategy for de novo engineering of ectopic autologous cartilage (EAC) within the subperiosteal space (*in vivo* bioreactor) in 2010, through the mere introduction of a biocompatible agarose gel that might promote hypoxia-mediated chondrogenesis. This study demonstrated that large volumes of bone could be engineered de novo within the *in vivo* bioreactor without cell implantation and administration of growth factors. In 2011, Gonçalves et al. [70] seeded goat bone marrow cells (GBMCs) into starch-polycaprolactone fiber mesh scaffolds and cultured in a flow perfusion bioreactor for up to 28 days using culture medium supplemented with transforming growth factor-β1, to investigate the chondrogenic differentiation of GBMCs under flow perfusion culture conditions. The histological staining and immunocytochemistry analysis shown the enhancement of mRNA expression for chondrogenic genes and ECM production under flow perfusion culture conditions. This study demonstrated that the flow perfusion system could create a favorable environment to promote the chondrogenic differentiation of GBMCs cultured onto starch-polycaprolactone fiber meshes. Compression stimulation could modulate the function of articular chondrocytes. However, the relevant studies were incomplete due to the lack of cell culture facilities capable of conducting experiments in a high-throughput, accurate and cost-effective manner. In 2014, Lin et al. [112] investigated the stimulating frequency effect of compressive loading on the functions of articular chondrocytes by a perfusion microcell culture system. The results demonstrated that the metabolic activity of chondrocytes was significantly affected by the stimulating frequency at the higher compressive strain of 40%. To develop a testing system to assess biomaterials for implants, which could permanently replace damaged cartilage and withstand the mechanical forces long term, Vainieri et al. [97] established a novel *ex vivo* osteochondral defect culture model in a mechanically stimulated microenvironment, which mimics the complex motion of an articulating joint *in vivo*. The results demonstrated that multidirectional loading, consisting of axial compression and ball oscillations, promoted maintenance of chondrocyte phenotypes through up-regulation of cartilage gene expression. In 2020, Yan et al. [113] investigated the cellular processes and

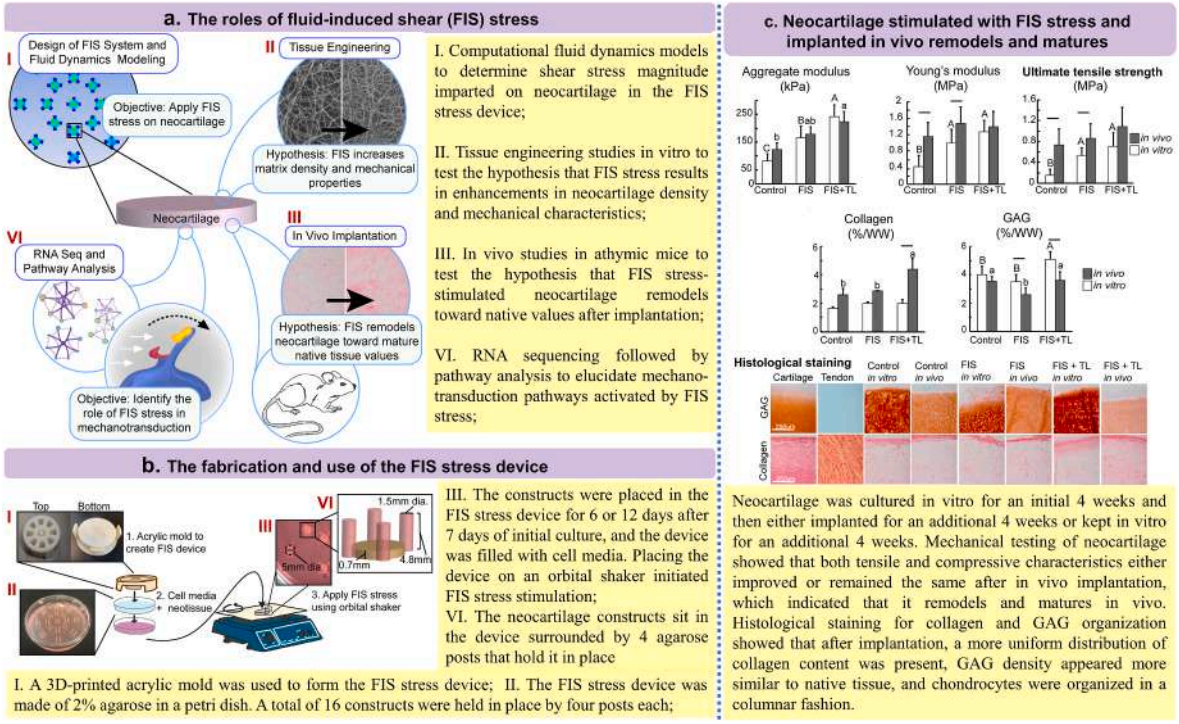

Fig. 8. The schematic diagram of a tunable device that applies fluid-induced shear (FIS) stresses to self-assembled neocartilage by using an orbital shaker to create oscillatory fluid motion: a) Overview of series of studies to elucidate the roles of fluid-induced shear (FIS) stress; b) The fabrication and use of the FIS stress device; c) Neocartilage stimulated with FIS stress and implanted *in vivo* remodels and matures. Reproduced with permission [114]: copyright 2020, IOP.

mechanism of exosomes produced by conventional 2D culture and exosomes produced from 3D culture of umbilical mesenchymal stem cells (U-MSCs) in a hollow-fiber bioreactor for the treatment of cartilage repair. The results showed that exosomes produced from 3D culture of U-MSCs in a hollow-fiber bioreactor increased the number and enhanced the functions of chondrocytes by stimulating cell proliferation, migration, and matrix synthesis, and inhibiting apoptosis. To understand how shear stress works and determine optimal levels of shear stress for neo-cartilage formation, Salinas et al. [114] designed a tunable device that applies fluid-induced shear (FIS) stresses to self-assembled neocartilage by using an orbital shaker to create oscillatory fluid motion (Fig. 8). An optimal range of FIS stress, 0.05–0.21 Pa, resulted in up to 3.6-fold improvements in mechanical properties of neocartilage *in vitro*. Implanted neocartilage treated with a combination of FIS stress and bioactive factors remodeled and matured *in vivo*, indicating that fluid-induced shear stress as a potent mechanical stimulation strategy in tissue engineering contributed to the formation of neo-cartilage.

5.2. Bioreactor application in integrated bi-layered osteochondral construct (strategy B)

Considering the different biochemical and biomechanical characteristics of cartilage and bone tissue, the bioreactor with different culture media and precise mechanical stimuli simultaneously ensured that each part of the osteochondral construct exhibited site-specific properties that could induce and maintain osteogenesis and chondrogenesis in the corresponding area.

Chang et al. designed an innovative double-chamber stirred bioreactor to engineer composite osteochondral constructs, as shown in Fig. 4a, [115]. Double-chamber stirred bioreactor is separate by silicon septum where the central part of the biphasic scaffold is fixed, so that the cartilage and bone layers of biphasic scaffold can be cultured independently within its respective chamber. Chang et al. [115] have constructed a tissue-engineered osteochondral construct from a novel biphasic scaffold. The sinbone block, also known as calcium-phosphate block made from calcined bovine bone, was soaked in the gelatin solution for 30 min, and the gelatin solution diffused into the sinbone block due to the capillarity effect. The integration of the cartilage part (porous gelatin) and bony parts (sinbone block with trabecular pattern) of the scaffold depends on the infiltration of the gelatin solution into the pores of the calcium phosphate. The biphasic scaffolds were seeded with porcine chondrocytes and cultured in a double-chamber bioreactor for 2 or 4 weeks. After 4 weeks of culture, the porcine chondrocytes retained their phenotype as proven immunohistochemically, and hyaline-like cartilage with lacuna formation could be clearly seen in the gelatin scaffold. These results suggested that biphasic scaffold had potentials for further application in osteochondral tissue engineering. For the dynamic bioactivity tests of bi-layered chitosan-based scaffolds *in vitro*, Malafaya et al. [116] also designed a double-chamber bioreactor, aiming at a future osteochondral application. In 2014, Lin et al. [117] fabricated a multi-chamber bioreactor and fitted into a microfluidic base to generate and maintain osteochondral constructs derived from human BMSCs. Specifically, hBMSCs-derived constructs were fabricated *in situ* and cultured within the bioreactor. When the osteochondral construct was inserted, two chambers were formed on either side of the construct that was supplied by different medium streams, in which cartilage and bone tissues developed and matured in tissue-specific microenvironments. This microfluidic-based multi-chamber bioreactor could offer novel capabilities for investigating the pathogenic mechanisms of osteoarthritis, physiology of osteochondral tissue, serving as a high-throughput platform to test potential disease-modifying osteoarthritis drugs (DMOADs). In 2019, Vukasovic et al. [118] developed the bilayer biomimetic osteochondral scaffold: the top layer of the scaffold consisted of equine type I collagen, and the bottom layer was made of a mineralized blend of type I collagen and Mg-HA, mimicking the structure and biochemistry of cartilage and subchondral bone. The cartilage digestion bioreactor automates and controls the cartilage tissue digestion phase, and the Tissue Culture Under Perfusion (T-CUP) bioreactor controls the ovine chondrocytes seeding, 3D proliferation and differentiation phases. The digestion bioreactor and T-CUP bioreactor were installed within a

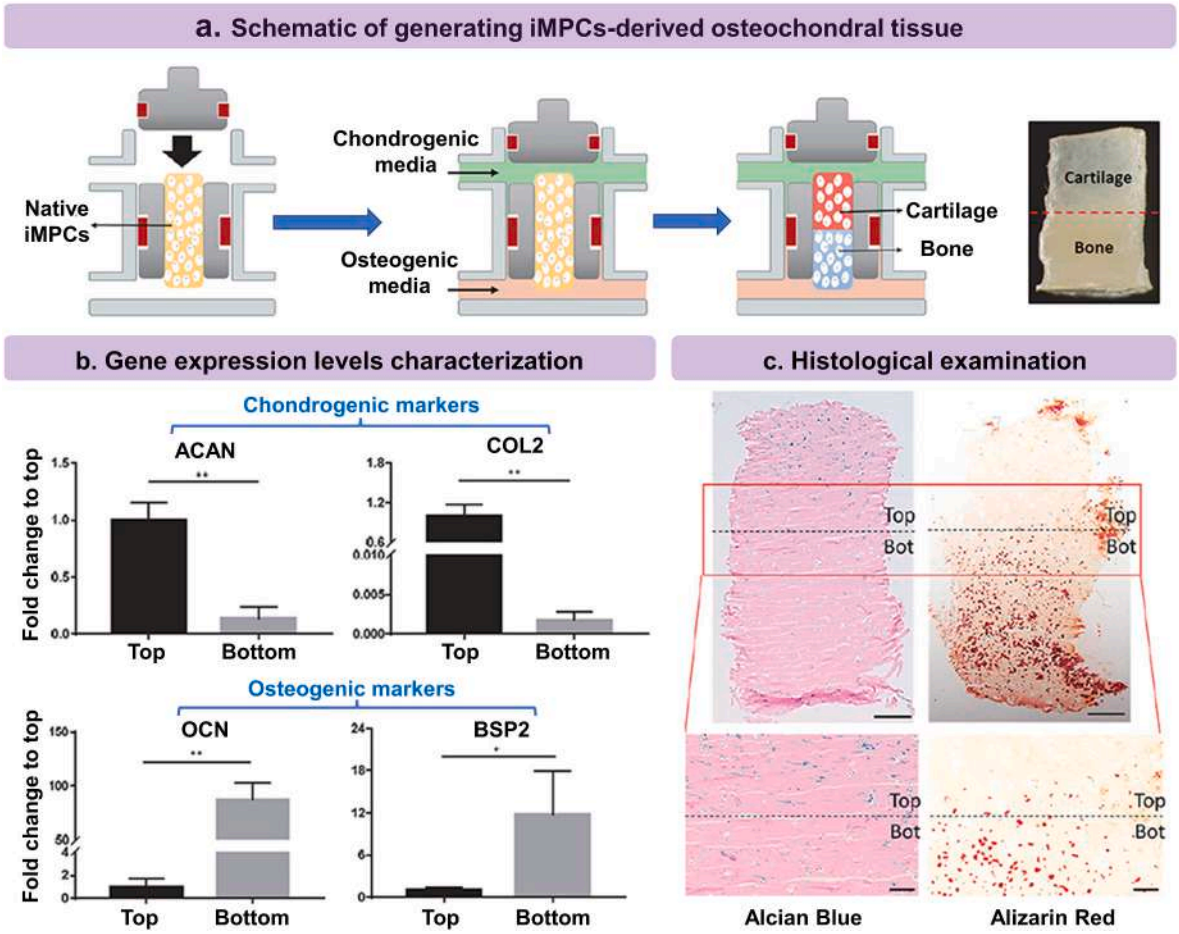

Fig. 9. Fabrication of iMPCs-derived osteochondral tissue in the dual-flow perfusion bioreactor: a) Schematic of generating osteochondral tissue containing cartilaginous and osseous layers; b) After 28-days differentiation, characterization of the engineered osteochondral construct in terms of the expression levels of chondrogenic markers (ACAN, COL2) and osteogenic markers (OCN, BSP2); c) Histological examination of the biphasic osteochondral tissues as regards the deposition of the tissue-specific matrix by Alcian Blue and Alizarin red stainin. Reproduced with permission [119]: copyright 2019, Frontiers.

declassified cleanroom at the Good Manufacturing Practice (GMP) facility. By demonstrating the safety and efficacy of bioreactor-generated grafts in two large animal models, all data suggested that bioreactor-manufactured grafts accelerated the repair of acute osteochondral defects compared to cell-free scaffold implants, and may result in a more robust repair in the longer term. This work represented a pivotal step towards implementing the bioreactor-based manufacturing system for the production of human osteochondral grafts for clinical applications. Lin et al. [119] have successfully developed an *in vitro* osteochondral tissue chips based on human mesenchymal progenitor cells (iMPCs) to model the pathologies of osteoarthritis (Fig. 9). Firstly, they optimized the chondro- and osteo-inductive conditions for iMPCs. Then iMPCs were encapsulated into photocrosslinked gelatin scaffolds and cultured using a dual-flow bioreactor with both chondrogenic and osteogenic media streams. Subsequently, the optimized chondrogenic and osteogenic media were perfused into the bioreactor via the top and bottom flows, respectively, to induce the formation of the biphasic osteochondral tissue, with cartilage in the top and bone in the bottom (Fig. 9a). During culture, iMPCs were differentiated into chondrogenic and osteogenic lineages. After 28 days of culture, a microphysiological osteochondral tissue chips were successfully formed, and specific gene expression (chondrogenic markers licluding aggrecan-ACAN and collagen type 2-COL2, and osteogenic markers including osteocalcin-OCN and bone sialoprotein 2-BSP2) and matrix deposition (histological examination) validated chondral and osseous phenotypes (Fig. 9b–c) [119], suggesting that cartilage-like tissue of osteochondral was formed in the top part and bone-like tissue was formed in the bottom. Unfortunately, perfusion-based devices still lack the possibility for mechanical stimulation to the tissue-engineered constructs during dynamic culture. In order to develop the culturing system for biphasic native or engineered osteochondral tissues, Chiesa et al. [120] also designed a 3D printed dual-chamber bioreactor which potentially accommodate a variety of interface tissues and enables the precise study of tissue crosstalk by creating two independent microenvironments while maintaining the tissue compartments in direct contact.

Osteochondral constructs combined with bioreactor culture methods could contribute to the production of constructs that contained the necessary mechanical and/or biochemical properties for implantation [76]. Compared with simultaneously culturing the integrated bilayered scaffold, culturing the two layers of biphasic scaffolds separately may increase its versatility. However, the double-chamber bioreactor could ensure that each part of the osteochondral construct exhibited site-specific properties that could induce and maintain osteogenesis and chondrogenesis in the corresponding area.

6. Perspectives towards future development

Although some advanced bioreactors have been used to create osteochondral constructs, there is not a perfect protocol for the use of bioreactors in osteochondral tissue engineering, so further study is needed for bioreactor-based osteochondral tissue engineering.

Firstly, formation of osteochondral neo-tissues with sufficient

collagen type II, proteoglycan, and other ECM components, is essential to reconstruct the osteochondral tissues with restored functions. However, the complexity of physiology and microenvironment of osteochondral tissue establish a major challenge in fabricating tissue-engineered osteochondral constructs which could meet the demand of specific repair sites in specific patients. Most importantly, we need to deeply understand the mechanism and various variation in functional and structural properties of osteochondral defect. Factors such as possible changes in the metabolic pathway (oxygen and nutrients), alterations in the biological remodeling dynamics, and variations in collagen and mineral status and perhaps their spatial distribution are currently under investigation. Therefore, the improved multifunctional bioreactors capable of performing multiple microenvironmental cues simultaneously are an inevitable trend in the future, as they could reduce the risk of contamination and provide the necessary stimulation for each stage. Especially, the multifunctional bioreactors, which contain oxygen tension, mechanical loading regimes and electromagnetic stimuli that could be conducive to the growth of bone and cartilage, might be a most promising future development directions in this field.

As electroactivity plays a vital role in the physiological functions of live organisms, electrical stimulation has been identified as a promising nonpharmacological technique that could modulate the behavior of cellular network, restore critical functions and accelerate tissue healing *in vitro* and *in vivo* [121–123]. Electrical field has been proven to offer critical bioactive cues to enhance osteogenesis by stimulating migration, proliferation, alignment and differentiation of osteoblasts [124]. Piezoelectric materials, as a promising electrical stimulator, are capable of converting mechanical stress into electrical cues without using external power source and electrodes to directly stimulating the growth of surrounding cells and the maturation and reconstruction of new bone tissue [125–132]. So, the combination of piezoelectric materials and bioreactors is also a promising trend in the future. Further, since the load of growth factors or genes is one of main means to enhance the bioactivities, the development of ready-to-use high-performance bioreactor-based osteochondral construct for delivering those bioactive substances should be another indispensable effort point. Given that the final goal of osteochondral tissue engineering is to facilitate the regeneration of both bone and cartilage, it should be emphasized that at least two kinds of bioactive substances could be properly loaded into different parts of the scaffold and could be all released in a sustained fashion. Probably, growth factors combined with multifunctional bioreactor should be used to promote the formation of osteochondral tissue, provide appropriate mechanics to maintain different parts of osteochondral tissue formation, and inhibit signaling to prevent endochondral ossification. While most of these efforts are yet to be translated into clinic, this outcome is promising as osteochondral constructs combined with bioreactor culture methods are yielding improved constructs. Alternatively, bioartificial scaffolds based on bioreactor systems have attracted tremendous attention because the sacrifice of autogenous tissues could be avoided and the scaffolds could be functionalized through incorporating bioactive cues (electric fields, growth factors, or structural guidance) to facilitate therapeutic effects.

Finally, it is also known that microgravity is deleterious for bone, leading often to losses in total bone mass. The main challenge is how to design a bioreactor with suitable mechanics matching mechanical property of native osteochondral tissue. It would be helpful to develop new biomimetic engineered osteochondral tissues by combining mechanical finite element simulations, advanced manufacturing technologies of osteochondral construct and bioreactors that mimic the native microenvironment of native joint. Moreover, quantitative analysis and computational modeling of stresses and strains experienced by both normal tissues *in vivo* because of a variety of activities and engineered tissues in bioreactors, could lead to more precise comparisons of *in vivo* and *in vitro* mechanical conditioning, and help to determine potential regimes of physical rehabilitation that are most appropriate for the patient.

In addition to generate grafts, bioreactor-based engineered constructs *in vitro* could produce non-implantable constructs to serve as external organ support devices when a compatible donor is not readily available. Furthermore, tissue-engineered constructs based on bioreactors could provide reliable model systems that facilitate a basic understanding of structure-function relationships under normal and pathological conditions and have potential applications in the field of molecular therapy.

7. Conclusion

As the bioreactor culture system allows for homogeneous nutrient transfer and provides mechanical cues required for the tissue development, considerable progress has been made in bioreactor-based osteochondral tissue engineering in the last decade. Although direct perfusion may benefit both cartilage and bone regeneration, it is difficult to culture different phases of osteochondral constructs simultaneously due to variations in inflow rate and culture time. Therefore, a compartmental or multifunctional bioreactor that could satisfy the requirements of the cartilage, bone, and the osteochondral interface simultaneously might be an ideal choice for future osteochondral construct. With the advances in this technology, we believe that in 10–20 years' time, tissue-engineered osteochondral construct will be easily accessible in ordinary clinics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors acknowledge financial support from the National Natural Science Foundation of China (No. 32171345), the Hebei Provincial Natural Science Foundation of China (No. C2022104003), the Basic Scientific Research Funds of Hebei province (No. JYT2020013), the Fok Ying Tung Education Foundation (No. 141039), the Fund of Key Laboratory of Advanced Materials of Ministry of Education, the International Joint Research Center of Aerospace Biotechnology and Medical Engineering, Ministry of Science and Technology of China, and the 111 Project (No. B13003).

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