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Challenges in engineering osteochondral tissue grafts with hierarchical structures Ivana Gadjanski, Gordana Vunjak Novakovic

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Abstract

Introduction—A major hurdle in treating osteochondral (OC) defects are the different healing abilities of two types of tissues involved - articular cartilage and subchondral bone. Biomimetic approaches to OC-construct-engineering, based on recapitulation of biological principles of tissue development and regeneration, have potential for providing new treatments and advancing fundamental studies of OC tissue repair.

Areas covered—This review on state of the art in hierarchical OC tissue graft engineering is focused on tissue engineering approaches designed to recapitulate the native milieu of cartilage and bone development. These biomimetic systems are discussed with relevance to bioreactor cultivation of clinically sized, anatomically shaped human cartilage/bone constructs with physiologic stratification and mechanical properties. The utility of engineered OC tissue constructs is evaluated for their use as grafts in regenerative medicine, and as high-fidelity models in biological research.

Expert opinion—A major challenge in engineering OC tissues is to generate a functionally integrated stratified cartilage-bone structure starting from one single population of mesenchymal cells, while incorporating perfusable vasculature into the bone, and in bone-cartilage interface. To this end, new generations of advanced scaffolds and bioreactors, implementation of mechanical loading regimens, and harnessing of inflammatory responses of the host will likely drive the further progress.

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Keywords

Anatomical shape; Biomimetics; Osteochondral grafts; Tissue engineering

1. Introduction

Osteochondral (OC) defects are in most cases areas of articular injury or degeneration involving damage of both the cartilage and subchondral bone. OC defects can be classified as focal lesions and degenerative lesions¹. The former are well delineated, caused by physical macro- and micro-trauma, as well as by aging and diseases such as osteochondritis dissecans² and osteonecrosis³. The latter are usually caused by the most common joint disease worldwide – osteoarthritis⁴. OC defects have limited ability for spontaneous healing, mostly due to the avascular nature of cartilage and disturbed communication with subchondral bone. These defects can induce significant pain, diminish patients' mobility and affect their quality of life, a situation accompanied with a high economic burden⁵.

Current clinical treatments (debridement, bone marrow stimulation techniques, osteochondral autograft transplantation (OATS)/mosaicplasty⁶, osteochondral allograft transplant⁷) are being used mostly for the OC defects in the knee, but also in the talus⁸. These treatments can improve clinical symptoms, but the underlying pathology remains uncured. Some treatments, such as allograft transplantation, can cause immune rejection⁹.

Tissue engineering (TE) offers new options for treating OC defects, by growing biological substitutes of native osteochondral complexes, through the individual and combined use of cells, biomaterial scaffolds, and culture systems (bioreactors)¹⁰. A number of TE strategies, including the use of custom-designed¹¹ scaffolds, with and without cells, have been implemented for the treatments of OC defects, with various degrees of efficacy¹².

It remains a challenge to treat OC defects due to different healing abilities and different morphology and physiology of the two types of tissues involved - articular cartilage and subchondral bone¹³. *In vivo*, the two tissues are naturally complementing each other through the network of intricate mechanisms, formed during the process of endochondral ossification, composing the osteochondral unit with unique biomechanical properties¹⁴. In a defect, the osteochondral unit is disturbed and should be reconstituted in order to initiate repair and restore structural and physiological properties of all its layers.

Stratified osteochondral units are composed of the cartilaginous hydrogel-like layer containing water (70 to 80%), collagen II (50 to 75%), glycosaminoglycans (GAGs) (15 to 30%)¹⁵ and chondrocytes (1-10%)¹⁶. The cartilage layer can be further subdivided into non-calcified (superficial, middle, deep zone) and calcified cartilage. A thin line referred to as "tidemark", located at the bottom of the deep zone, marks the transition from noncalcified to calcified cartilage¹⁷.

Beneath the chondral phase is the porous subchondral bone, interdigitated with the cartilage and connected through the cement line. Subchondral bone is comprised of water, collagen I, hydroxyapatite (HA) and three cell types: osteoclasts, osteoblasts and osteocytes. Blood

vessels can reach out from the bone into the calcified cartilage, while microcracks and fissures further facilitate transfer of molecules^{18, 19} (**Fig. 1**).

The main approach to achieving such complex biological organization of cartilage interfaced with the bone is by recapitulating *in vitro* key aspects of the *in vivo* developmental processes. This approach, termed biomimetic TE, entails the use of cells (ideally the patient's own) that can differentiate into cartilage and bone cells. The cells are “instructed” to form an OC unit by coordinated use of a biomaterial scaffold (a structural and logistic template designed to provide structural and biological cues of the native osteochondral unit¹³) and bioreactors (designed to provide an controllable *in vivo* like cellular microenvironment - a cell niche¹⁰). The stratification is achieved through the multiphasic structure of the scaffold and implementation of gradients of factors. The communication between tissue layers¹⁹, integration of the interface between the chondral phase and osseous phase¹⁶ and dynamics of the tidemark²⁰ are significantly harder to mimic and remain to be some of the key challenges in OC defects treatment.

Osteochondral grafts are investigated with the aim of creating neotissues for potential clinical application, but also to serve as controllable models of high biological fidelity for studies of osteochondral tissue development using both the primary cells (chondrocytes, osteoblasts) and the stem cells derived chondro- and osteoprogenitors²¹. Osteochondral grafts can also serve as *in vitro* pre-clinical models for studies of disease pathology, identification of therapeutic targets, and evaluation of drug toxicity and efficacy^{22, 23}.

2. Biomimetic system component I: Cells

Cellular techniques for treating OC defects can be based on either primary cells (chondrocytes, osteoblasts) or mesenchymal stem cells, with or without scaffolds. Excellent reviews on cellular techniques such as ACI (autologous chondrocyte implantation), chondrospheres and MACI (Matrix-induced autologous chondrocyte implantation) are available^{24, 25}. Here we focus on cellular components of hierarchical osteochondral (OC) grafts.

Most investigated stem cells for growing OC grafts are adult mesenchymal stem/stromal cells (MSCs), because of their potential to undergo both chondrogenesis and osteogenesis. MSCs are historically obtained from bone marrow aspirates (BMSCs)²⁶, and more recently from other tissue sources: adipose tissue²⁷ (adipose-derived stem cells - ADSCs), amniotic fluid²⁸ (AFSCs), synovium^{29, 30} and periosteum³¹. The use of peripheral blood has also been reported clinically, both for obtaining stem cells³² and progenitor cells³³. Recently, multipotent adult progenitor cells (MAPC) are gaining more attention, as potentially better candidate seed cells for OC grafts. Bone-marrow-derived hMAPCs were differentiated *in vitro* into cells expressing chondrocyte markers, but their morphology remained different from that characteristic for chondrocytes³⁴.

Human induced pluripotent stem cells (hiPSCs) have also demonstrated significant potential for cartilage regeneration. Undifferentiated hiPSCs can be expanded through high number of passages, whereas chondrocytes and most adult stem cells such as MSC and ADSC show decreasing proliferation and differentiation potential already after 4 passages in culture³⁵.

The application of hiPSC is limited by the current protocols for chondrogenic differentiation that are complicated and inefficient primarily due to the need for intermediate embryoid body formation, required to generate endodermal, ectodermal, and mesodermal cell lineages.

Recently, Nejadnik et al. reported a new, straightforward approach for chondrogenic differentiation of hiPSCs, which avoids embryoid body formation³⁶, and instead is driving hiPSCs directly into mesenchymal stem /stromal cells (MSC) and chondrocytes. hiPSC-MSC-derived chondrocytes showed significantly increased expression of chondrogenic genes compared to hiPSC-MSCs. Following transplantation of hiPSC-MSC and hiPSC-MSC-derived chondrocytes into osteochondral defects of arthritic joints of athymic rats, MRI studies showed engraftment, and histological correlations showed the production of hyaline cartilage matrix. De Peppo et al. engineered functional bone substitutes by culturing hiPSC-derived mesenchymal progenitors on osteoconductive scaffolds in perfusion bioreactors, and confirmed their phenotype stability in a subcutaneous implantation model³⁷.

Human embryonic stem cells (hESCs) are also an attractive candidate for cell replacement therapy because of their unlimited self-renewal and ability for differentiation into mesodermal derivatives as well as other lineages. There is a number of protocols for inducing osteogenic and chondrogenic differentiation of the hESCs through embryoid bodies (EBs)³⁸, by co-culture/conditioned culture with fully differentiated chondrocytes³⁹, MSCs⁴⁰, ESC-derived MSCs⁴¹ or by directed differentiation to chondro- and osteogenic cells^{42, 43}. Synergistic effects of hypoxic conditioning and morphogenetic factors are also investigated in detail in the context of generating chondrocytes/osteoblasts from hESCs and hMSCs. Yodmuang et al. showed that chondrogenesis in hESCs can be synergistically enhanced by controlling oxygen tension and morphogenic factors secreted by chondrocytes⁴⁴. In their directed differentiation protocol, Oldershaw et al. demonstrated that hESCs progress through primitive streak or mesendoderm to mesoderm, before differentiating into a chondrocytic cell aggregates⁴³.

In our previous review on time-dependent processes in stem cell-based tissue engineering of articular cartilage⁴⁵, we pointed out that tissue engineering strategies recapitulating some temporal aspects of native development, may be more successful than those that disregard the temporal control of tissue formation. We also remarked that it appears likely, based on the results by Oldershaw and others, that in order to increase the efficiency of chondrogenesis from hESCs one must direct differentiation of hESCs first into the MSC-like phenotype and allow mesenchymal cell condensation (pre-cartilage condensation) to take place⁴⁵.

This notion is further confirmed by two recent studies from our group^{46, 47}. Bhumiratana et al. report that clinically sized pieces of human cartilage with physiologic stratification and biomechanics can be grown *in vitro* by recapitulating some aspects of the developmental process of mesenchymal condensation. By exposure to transforming growth factor- β (TGF- β), MSCs were induced to condense into cellular bodies, undergo chondrogenic differentiation, and form cartilaginous tissue, in a process designed to mimic mesenchymal condensation leading into chondrogenesis.

We discovered that the condensed mesenchymal cell bodies (CMBs) formed *in vitro* set an outer boundary after 5 days of culture, as indicated by the expression of mesenchymal condensation genes and deposition of tenascin. Before setting of boundaries, the CMBs could be fused into homogenous cellular aggregates, without using a scaffolding material, giving rise to well-differentiated and mechanically functional cartilage. The formation of cartilage was initiated by press-molding the CMBs onto the surface of a bone substrate (**Figure 2A-C**). By image-guided fabrication of the bone substrate and the molds, the osteochondral constructs were engineered in anatomically precise shapes and sizes (**Figure 2D**).

After 5 weeks of cultivation, the cartilage layer assumed physiologically stratified histomorphology, and contained lubricin at the surface, proteoglycans and type II collagen in the bulk phase, collagen type X at the interface with the bone substrate, and collagen type I within the bone phase⁴⁶. For the first time, biomechanical properties of cartilage derived from human MSCs were comparable to those of native cartilage, with the Young's modulus of >800 kPa and equilibrium friction coefficient of <0.3.

We also demonstrated that CMBs have capability to form mechanically strong cartilage–cartilage interface in an *in vitro* cartilage defect model. The CMBs, which acted as “Lego-like” blocks of neocartilage, were capable of assembling into human cartilage with physiologic-like structure and mechanical properties⁴⁷. This method could be highly effective for generating human osteochondral tissue constructs, and for repairing focal cartilage defects to replace currently used dissociated chondrogenic cells.

Taken together, these results support the benefits of scaffold-less techniques of tissue engineering for generating self-assembling tissues⁴⁸. Without a scaffold to interrupt cell–cell signaling and stress shielding, cells are more able to respond to stimuli, secrete and assemble cartilaginous matrix, and bond with the surrounding tissue²⁴.

3. Biomimetic system component II: Scaffold

While the scaffold-less techniques are gaining interest for certain applications, scaffolds are critically important for OC tissue engineering. An OC scaffold should be biocompatible (non-toxic, non-immunogenic), have sufficient mechanical integrity for habitual loading, have ability for guiding the formation of cartilage, bone and an interface between these two tissues in a spatially defined manner, and degrade at a rate matching that of tissue formation. In order to support the formation of an osteochondral unit, it is useful to design scaffolds with stratified molecular, structural and mechanical properties, by using composites of biomaterials. The scaffold material can also be functionalized with attached or encapsulated growth factors to enhance tissue development. The size and shape of the scaffold ideally should match the anatomy of the specific implantation site¹¹.

3.1 Scaffold biomaterials

There is a range of biocompatible materials currently used for OC grafts, from natural to synthetic polymers, inorganic (ceramics and bioactive glasses) and metallic materials. As there is no material that can meet the necessary requirements for the formation of a complex,

stratified tissue, the prevalent approach is to use multicomponent systems and hybrid scaffolds⁴⁹. Natural polymers include proteins (e.g., collagen, gelatin, silk)⁵⁰, fibrin, polysaccharides (chitosan or starch), alginate, hyaluronic acid, chondroitin sulphate, and polyesters⁵¹. In general, natural polymers have a number of advantages for use in OC grafts, as they can provide the necessary biological cues to the attached cells, mimic the native extracellular matrix (ECM), and offer excellent biocompatibility and degradability, along with the low cost and high availability. However, they can also have limitations, such as weak mechanical properties and batch-to-batch variability⁵².

Synthetic polymers, such as polyglycolic acid, polylactic acid, polycaprolactone - PCL, poly(L-lactic-co-glycolic acid) - PLGA, polydioxanone, poly(propylene fumarate), polyorthoesters, polyphosphazenes, and polyanhydrides have characteristics almost exactly complementary to those of native biomaterials. Synthetic materials can be easily tailored to the target application – e.g. their degradation and mechanical properties can be modified by varying the conditions of synthesis and processing^{53, 54} and the material properties can easily be standardized. However, most synthetic polymers are hydrophobic (causing problems with cell attachment), and lacking regulatory molecules and interaction sites present in the native ECM (causing problems with cell differentiation).

Blends of hydrophobic and hydrophilic polymers are sometimes used to enhance hydrophilicity and promote cell attachment⁵⁵, and the surfaces are being functionalized by bioactive molecules to induce and support cell function^{56, 57}. Blends of bioceramics/bioglass and synthetic or natural polymers can also provide improved physicochemical and biological properties⁵⁸ and enable fabrication of scaffolds with desired mechanical characteristics^{59, 60}. In general, hybrid scaffolds consisting of a synthetic component providing mechanical strength and natural component providing biological cues show great promise for use in osteochondral tissue engineering^{61, 62}.

A novel hybrid scaffold for bone regeneration was developed by Tampieri et al⁶³ using nano-apatite, self-assembling collagen, and magnetite nano-particles. The magnetic phase acted as a cross-linking agent for collagen, and allowed control of scaffold porosity. Gradients of bio-mineralization and attached bioactive factors were established for osteochondral applications, with the aid of an external magnetic field.

After testing the physical, chemical, structural, biological and mechanical properties of a scaffold *in vitro*, it is necessary to evaluate its *in vivo* performance under conditions mimicking those in an implanted OC unit. *In vivo* models of osteochondral defects (OC defects) are mostly developed for rodent models, which allow experimentation at a low cost and with large sample sizes. However, the graft sizes, healing times, the conditions of loading and the inflammatory/immune environment are quite different from those in human. An alternative approach was proposed where osteochondral cores from large animals are implanted subcutaneously in rats for high-throughput screening of multiphase scaffold designs and tissue-engineered constructs⁶⁴. An excellent overview of recent *in vivo* studies on OC tissue engineering can be found in a recent review by Yan et al⁶⁵.

Injectable hydrogels are particularly convenient materials for *in vivo* applications. An emerging class of bioinspired polymers for cartilage and bone tissue engineering are glycopolypeptides that mimic naturally occurring glycoproteins, that have been processed into injectable hydrogels, by enzymatic crosslinking of glycopeptides in the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2)⁶². These hydrogels have controllable physicochemical properties (such as gelation time, storage modulus, swelling, degradation time), by simply varying the concentrations of HRP and H_2O_2 during hydrogel preparation. When injected subcutaneously into rats, the glycopolypeptide hydrogels rapidly form *in situ*, and exhibit acceptable biocompatibility. When used *in vitro* to encapsulate chondrocytes, these hydrogels supported cell proliferation and the production of GAG and type II collagen⁶⁶.

3.2 Scaffold designs

Efficient OC scaffold design should provide hierarchical structure, desired mechanical and mass transport properties (stiffness, elasticity, permeability, diffusion), and ability for processing into precise anatomical shapes. In this context, hierarchy is important at all levels - from nano, to micro to macro, in order to meet the often conflicting requirements for mechanical function, mass-transport, and biological regulation⁶⁷.

3.2.1 Gradient-based architectures—Hierarchical structures of tissue constructs involve gradients of multiple properties: physical (via scaffold design) and biochemical (differentiation factors, cell adhesion molecules)⁶⁸.

The first types of OC scaffolds were composed of only one type of biomaterial, with uniform porosity and architecture, and used a single cell type, without variation of the local biological environment⁶⁹. Such lack of spatial variation limited the potential of monophasic scaffolds to provide effective templates for osteochondral tissue engineering.

Grayson et al. showed the importance of the spatial regulation of human MSC differentiation in engineered osteochondral constructs, by using biphasic constructs made by interfacing agarose gels and bone scaffolds, with both compartments seeded with the same bone marrow-derived hMSCs⁷⁰ (**Fig. 3**). The study investigated the combined effects of three sets of regulatory factors: (i) cell pre-differentiation, (ii) medium supplements, and (iii) perfusion rate of medium through the scaffold on spatial control of hMSC differentiation. Bone-marrow derived hMSCs were expanded in their undifferentiated state, seeded into biphasic scaffolds and cultured for 5 weeks in a bioreactor with perfusion of medium through the bone region, with corresponding static cultures to serve as a control.

For the cartilage compartment, static culture of undifferentiated hMSCs in chondrogenic medium elicited the best chondrogenic responses, while perfusion culture of pre-differentiated osteoblasts or undifferentiated hMSCs with cocktail medium elicited the best osteogenic responses. One result of this study was that an osteochondral construct should consist of two discrete compartments, each enabling the cultivation of optimal cell types and exposure to optimal stimuli, including medium compositions, flow conditions⁷⁰. To this end, a bioreactor system is needed that can support the cultivation of such a biphasic construct. Another result is that biphasic constructs lead to the formation of an interface that

is different from that in a native tissue. In the clinical setting, many biphasic approaches have not been successful. One example is the TrueFit plug, biphasic commercially available and licensed biomaterial implant for treating the chondral and osteochondral defects of the knee⁷¹. A very recent review of the current literature by Verhaegen et al. that investigated clinical, radiological, and histological efficacy of the TruFit plug in restoring osteochondral defects in the joint, concluded that data do not support superiority or equality of TruFit compared to treatments by mosaicplasty or microfracture⁷².

Therefore, additional scaffold properties (such as graded molecular composition, structure, and biomechanics) appear necessary for recapitulating the native interface and the heterogenous cell-cell communication between the cartilage and bone⁷⁰.

Current state-of-the-art in the design of stratified or gradient scaffold is to mimic the structural, mechanical, and biochemical microenvironment of a native osteochondral unit. There are multiple ways to achieve stratification and gradient-based composition. One simple approach is to build composite scaffolds through multilayered scaffold design, to generate structural templates for the cartilaginous layer, the tidemark and calcified cartilage, and the subchondral bone, while allowing the transitional interface layer to efficiently connect cartilage and bone. Such complex but necessary structure is usually accomplished by using two or more different materials. Integration between layers and with the native tissue is achieved by suturing⁷³, cell-mediated ECM formation, use of fibrin and other glues,¹¹ or by simple press fitting⁶⁴. Highly meritorious reviews on multiphasic scaffolds were recently published by Jeon⁶⁹, Nukavarapu¹⁷ and Yousefi⁷⁴.

Human clinical trials with triphasic OC grafts reported by Kon et al⁷⁵ offered good clinical outcomes at the midterm follow-up⁷⁶. The scaffold used in this clinical trial was a cell-free three-gradient multilayer structure consisting of (i) a lower layer of a 30/70 blend of biomineralized type I collagen (Coll-I) and hydroxyapatite (HA) corresponding to the subchondral bone; (ii) an upper layer of collagen, mimicking the cartilaginous region; and (iii) an intermediate layer of biomineralized collagen, consisting of a 60/40 blend of Coll-I and HA, resembling the tidemark^{75, 77}.

Currently, the most used treatment approach to joint repair in the preclinical setting is a scaffold/cell combination, with MSCs being the favored cell type¹². Bone marrow aspirates have been the most used source of MSCs, but other sources such as lipoaspirates and synovium are gaining interest. In studies comparing the cartilage and bone tissue outcomes using scaffolds with or without cells, a majority of studies reported superior results with the use of cells (71 of 89 recent studies)¹². In clinical settings, most frequently used were chondrocyte-seeded scaffolds, with only 7 studies using MSC-seeded scaffolds, with cells derived from bone marrow.

Technical and regulatory difficulties in managing cell cultures, along with the development of a new generation of materials able to exploit the intrinsic tissue regeneration ability, motivate the clinical use of cell-free scaffolds¹². However, even though cell-free scaffolds might show promising results, they often result in incomplete repair of cartilage and subchondral bone⁷⁸.

Published work shows that the structural stratification alone is not sufficient for establishing effective transition between two tissues as different as cartilage and bone, prompting the need to also establish biochemical gradients, particularly in the interface region⁷⁹. Such biochemical gradients can be achieved by embedding the growth factors, non-growth factor inductive agents (e.g., hydroxyapatite) and other signaling molecules (therapeutic drugs, genes) into the scaffold.

Biomaterial-based scaffold formulations (3D porous matrix, nanofibre mesh, hydrogels and microspheres) are the major components that are used to deliver the bioactive molecules⁸⁰. For example, Dormer et al. distributed microspheres loaded with chondrogenic (TGF- β 1) and osteogenic (BMP-2) factors into the two regions of a PLGA scaffold, to produce opposing growth-factor gradients for the formation of cartilage and bone⁸¹. Therapeutic molecules can be surface-tethered to the microspheres⁶². Using “raw materials” i.e. components like chondroitin sulfate and bioactive glass in 3D scaffolds was suggested for establishing continuous gradients of material composition and signaling⁸². At this time, the best approach seems to be to couple biochemical and structural gradients towards achieving native-like architecture and integration⁸³.

3.2.2 Microstructural design—Besides gradient composition, establishing proper pore configurations is another basic criterion in scaffold design. The size and geometry of scaffold pores and the overall porosity regulate cell infiltration and attachment, vascularization (particularly in the osseous part of the OC construct) and remodeling processes (tissue buildup and scaffold degradation). Notably, the measured effects of porosity on osteogenesis were different under *in vitro* and *in vivo* conditions. *In vitro*, low porosity was shown to stimulate osteogenesis, presumably by suppressing cell proliferation and forcing cell aggregation, whereas, *in vivo*, a greater rate of bone formation was achieved using highly porous scaffolds with large pores. However, such scaffolds have poor mechanical properties, thereby setting an upper functional limit for the pore size and porosity.

Pores of 400 μ m are recommended by most groups, for enhancing new bone formation and the formation of capillaries, and the minimum pore size of ~100 μ m, as smaller pores limit cell migration and mass transport^{84, 85}. In addition to an appropriate porosity, it is important to provide strut dimensions between the interconnecting pores, as to maintain the mechanical strength of the scaffold, and allow penetration of cells and nutrients throughout the scaffold volume^{85, 86}. An excellent review of the roles of porosity and pore size in tissue-engineered scaffolds was recently published by Loh et al⁸⁷.

The pore structure depends primarily on scaffold fabrication. Common methods include fiber bonding, solvent casting/particulate leaching, gas foaming, freeze-drying and phase separation⁸⁸. Porous scaffolds processed using these techniques have controlled pore size and porosity⁴⁹. However, these conventional methods are not sufficiently precise for achieving complex scaffold internal architecture and shapes⁸⁷. In addition, these methods use toxic solvents that are not easy to completely remove.

Over the last several decades, computer-aided design and manufacturing (CAD/CAM) technologies have advanced to the level where it is possible to customize scaffolds to meet the needs of the patient, using scanned images of the defect. Lately, a new term is being introduced, Computer-Aided Tissue Engineering (CATE). CAM techniques are particularly important for achieving hierarchical and functionally graded scaffolds, by incorporation of micro- and nano-scale features that can improve both the mechanical properties and tissue regeneration, through toughening mechanisms and better cell adhesion, respectively⁶⁷.

Electrospinning is becoming a method of choice for production of nanofibers that are similar to the fibrous components of native ECM⁸⁹. Large surface areas of electrospun nanofibers and their porous structure enable efficient cell adhesion, proliferation, migration, and differentiation^{57, 90}. The nanofibers can be further functionalized by incorporating bioactive species⁹¹⁻⁹³. This technique has some limitations such as the charge build-up during processing that prevents fiber deposition and can limit the thickness of the final scaffold. To overcome this, Vaquette et al. combined one of the conventional methods - thermally induced phase separation (TIPS) with electrospinning and obtained thick and strong scaffolds⁹⁴.

Yang et al. report bone generation *in vivo* on 3D electrospun fibers, via endochondral pathway⁹⁵. Using wet-electrospinning system, they produced scaffolds consisting of loose and uncompressed nanofibers. Rat bone marrow cells were seeded on these scaffolds, chondrogenically differentiated *in vitro* for 4 weeks and subcutaneously implanted for 8 weeks. The cells infiltrated into the scaffolds and deposited abundant cartilage matrix during chondrogenic priming *in vitro*. This cartilage template subsequently remodeled into bone, following implantation⁹⁵.

3.2.3 Anatomically shaped scaffolds—One approach to generating anatomical hierarchical image-based scaffold architectures, i.e. designer custom-tailored scaffolds, is to use additive (layer-by-layer) manufacturing (AM) processes^{11, 67}. Rapid prototyping via 3D printing⁹⁶ yields better control of the scaffold architecture, both on the micro- and macroscale, which is important for manufacturing of hierarchical scaffolds. Precise reconstruction of the native tissue geometry achieved via 3D printing is particularly important for large and complex OC grafts that are engineered to replace whole joint sections, because of the need to also reproduce proper joint mechanics during articulation with opposing surfaces¹¹.

In general, AM systems may be categorized based on the way the materials are deposited: (i) laser-based machines that either photo-polymerize liquid monomer (stereolithography) or sinter powdered materials (selective laser sintering), and (ii) powder-based free-form fabrication using “inkjet” printing and extrusion (fused deposition), with either thermal or chemical processing of the material as it passes through a nozzle. All of these modalities can be utilized for fabrication of the porous hierarchical scaffolds with complex architecture and anatomical shapes^{67, 74, 87, 97, 98} that will be subsequently seeded with cells.

Recent advancements in AM technologies allowed for the direct incorporation of the live cells in the scaffold fabrication process – jointly referred to as biofabrication or 3D

bioprinting⁹⁶. Similar to the AM techniques for scaffold fabrication, there are three major types of 3D bioprinting techniques that are currently available: (i) inkjet bioprinting^{99, 100}, (ii) microextrusion bioprinting⁹⁵, and (iii) laser-assisted bioprinting^{97, 101}.

In an interesting study Levato et al. combined bioprinting and microcarrier technology to fabricate bilayered osteochondral models of clinically relevant size (16 mm × 5 mm × 5 mm) using two different bioinks. For the bone compartment, the bioink consisted of PLA microcarriers loaded with rat MSCs that were encapsulated in gelatin methacrylamide-gellan gum (GelMA-GG). The cartilage layer was printed using bioink made of the MSCs in GelMA-GG, without microcarriers⁹⁷. Microcarrier encapsulation improved the compressive modulus of the hydrogel constructs, facilitated cell adhesion, and supported osteogenic differentiation and bone matrix deposition by MSCs.

However, the 3D bioprinted constructs generally lack the structural integrity and mechanical properties needed for the use *in vivo*, limiting their utility for repairing load-bearing tissues, such as cartilage. To overcome this limitation, hybrid systems were introduced, that combine multiple processing methods. One example is the alternation of electrospinning of PCL fibers with 3D inkjet printing of rabbit chondrocytes in fibrin–collagen hydrogel, that resulted in 1 mm thick, five-layer tissue constructs¹⁰². In another study, several techniques (rapid prototyping, impression molding, injection molding) were integrated into a step-by-step framework for fabricating large osteochondral constructs with correct anatomical architectures and topologies for restoring the articular surfaces of diarthrodial joints¹⁰³.

4. Biomimetic system component III: Bioreactor

To support the maintenance of differentiated cell phenotypes and promote construct maturation, a bioreactor is necessary to provide environmental control, exchange of nutrients and metabolites, and biophysical signaling. For metabolically active tissues such as bone, an engineered construct cultured statically in a well plate would develop only a 100-200 µm thick outer layer of healthy viable tissue, around a sparsely populated inner region. A key requirement for bioreactor cultivation of tissues that are normally vascularized is the interstitial flow of culture medium through the tissue space, that facilitates exchange of nutrients – and most critically oxygen, metabolites are regulatory factors to and from the cells, over minimal diffusional distances. Ideally, the medium would be perfused through a network of endothelialized channels serving as precursors of the vascular network that will connect to the blood supply of the host. Such bioreactor systems are biomimetic in nature, as they provide convective-diffusive mass transport similar to that between blood and tissue, along with dynamic hydrodynamic shear that is an important regulatory factor for bone development and wellbeing. For cartilage, a biomimetic approach would involve dynamic compression that provides both the mechanism for fluid transport through the tissue and the necessary biophysical stimuli.

4.1 Bioreactors for engineering anatomically shaped osteochondral tissues

To engineer anatomically shaped constructs, the bioreactor needs to be customized to accommodate the specific geometry of the forming tissue, direct fluid flow through (and not around) the tissue, and provide gradients of physical and molecular cues controlling the

spatial and temporal patterns of cell differentiation and assembly¹¹. Such a specialized bioreactor should also provide a way to monitor the communication between chondrocytes and osteoblasts across the osteochondral junctions²². In general, a biomimetic bioreactor-scaffold system should recapitulate as many as possible aspects of the native tissue environment.

A bioreactor for engineering osteochondral tissues should in principle contain two discrete compartments – cartilage and bone, with an interface in between - designed to provide the cells in either region with an environment conducive and stimulatory for the formation of a specific tissue structure, including the provision of specific culture media⁷⁰. In the cartilage region, it is necessary to provide chondrogenic growth factors in combination with dynamic loading¹⁰⁴⁻¹⁰⁶, while in the bone phase the osteogenic growth factors should be combined with interstitial flow of medium to enhance nutrient transfer and provide shear stress^{37, 107}. In such a biomimetic bioreactor, the cartilage and bone phases are grown in apposition while each is being stimulated with adequate stimuli, in order to achieve native-like osteochondral integration.

To engineer anatomically shaped grafts, it is necessary to fabricate the bioreactor culture chamber in the exact shape of the graft to ensure a tight seal around the scaffold and ensure medium perfusion through the interstitial spaces within the construct rather than around the periphery (**Figure 4**)¹⁰⁸. Temple et al. describe the protocol to fabricate such bioreactor for cultivation of anatomically shaped human bone grafts¹⁰⁹.

Temporo-mandibular joint (TMJ), the only articulating joint in the head, has been a primary target for CATE of anatomically shaped scaffolds, because of the prevalence of TMJ disorders, a particularly complex and patient-specific anatomical configuration of the TMJ, and very high loading forces associated with its function¹¹⁰. Also, the small size of the TMJ, relatively to the larger joints such as knee or hip, reduces the diffusional limitations of nutrient transport to the cells¹¹.

Previous studies showed the potential of generating bone tissue by seeding osteoblastic cells onto 3D scaffolds and culturing them in osteoinductive medium¹¹¹. It was also shown that embryonic and adult stem cells can be differentiated into osteoblasts with the aid of biological and mechanical stimulation^{37, 41, 107}. The development of bioreactor systems capable of providing effective nutrient transfer to cells throughout the scaffold volume enabled the *in vitro* formation of large (centimeter-size) pieces of functional, living bone with homogeneously distributed cells and their ECM.

The method for engineering clinically sized, anatomically shaped, viable human TMJ grafts by using hMSCs and a biomimetic scaffold-bioreactor system that was developed by our lab¹⁰¹ has been recently expanded to engineering of large bones in the CMF region using 3D printing¹⁰². To this end, Temple et al. generated anatomically correct 3D PCL scaffolds of the maxilla and mandible, and evaluated the vasculogenic and osteogenic capacity of capacity of human adipose-derived stem cells (hASCs) seeded onto PCL sheets, both *in vitro* and *in vivo*. Taken together, these results support the feasibility of generating porous scaffolds that replicate the incredibly complex anatomies and internal architectures of the

mandible and maxilla. Furthermore, the anatomically shaped scaffolds maintained the same high porosity observed in the rectangular scaffolds, allowing for cell seeding and graft vascularization¹⁰².

This example illustrates how the scaffold and bioreactor complement each other in the biomimetic system, towards recapitulating the key aspects of the *in vivo* environment and providing support for the cell growth, matrix synthesis and tissue maturation. While the scaffold provides structural and logistic support for the seeded cells and conveys biochemical and biophysical signals, the bioreactor provides a controllable native-like environment for tissue formation, maturation and remodeling. A recent review provides an overview of bioreactors used in osteochondral tissue engineering.²²

5. Engineering osteochondral interface

Establishing the osteochondral interface with native-like zonal organization remains one of major challenges in OC engineering. To achieve proper interface, it is necessary to enable formation of the zone of calcified cartilage (ZCC) that separates a non-calcified cartilage and the underlying bone. In this region, the cells are hypertrophic chondrocytes and the extracellular matrix contains type II and X collagens, calcium deposits and vertically running fibers¹¹². ZCC has different mineral content from both the noncalcified cartilage and the underlying bone^{20, 113}.

Various approaches to achieve continuous gradients that exist at OC interface have been explored, with only limited success. Dormer et al. used microspheres for selective local delivery of bioactive signals in the OC construct⁸¹, while Grayson et al. attempted to form gradients in the bioreactor (two-compartment design)⁷⁰. However, the ZCC was not similar to the native one in either case. One of the reasons might be the use of scaffolds that limited tissue integration. If the rate of scaffold degeneration is not well adjusted to the rate of tissue accumulation it can happen that the scaffold collapses under load of the newly formed tissue, destroying the interface.

This was the reasoning used by Lee et al. to devise a scaffold-less two-stage protocol using sheep bone marrow MSCs, pre-differentiated to chondrocytes, harvested and then grown on a porous calcium polyphosphate (CPP) substrate in the presence of triiodothyronine (T3)⁴⁰. T3 was withdrawn, and additional pre-differentiated chondrocytes were placed on top of the construct and grown for 21 days. The authors stipulate that the T3 treatment may have affected tissue fusion and have induced the expression of hypertrophic chondrocyte makers. This protocol yielded two distinct zones: hyaline cartilage and calcified cartilage adjacent to the substrate. Constructs with the calcified interface had comparable compressive strength to native OC tissue (sheep) and higher interfacial shear strength compared to control without a calcified zone¹¹⁴.

St-Pierre et al. achieved ZCC formation in the constructs made of chondrocytes seeded on CPP substrate by application of the thin calcium phosphate film to the substrate surface. This prevented the accumulation of inorganic polyphosphates released from the CPP and associated inhibition of mineralization in the cartilaginous layer, and allowed the mineralized ZCC to form. The mineral in the ZCC was similar in crystal structure,

morphology and length to that in native articular cartilage. Generation of such ZCC led to a 3.3-fold increase in the interfacial shear strength of the whole construct¹¹³. However, both of these protocols are yet to be tested on the bone surface i.e. in the OC construct with both chondro- and osteogenic layers generated from the cells.

Cheng et al. report the *in vitro* formation of a MSC-derived osteochondral interface as an intact and continuous ZCC resembling the native counterpart¹¹². They used MSCs and collagen monomers to fabricate undifferentiated subunits (MSC-collagen microspheres), differentiated these naïve subunits into chondro- and osteogenic microspheres and aggregated them to form chondro- and osteogenic layers. The two layers were brought into contact with a middle undifferentiated MSC-collagen layer. The cells from this middle layer formed the ZCC¹¹². Very similar approach for OC interface forming by placing a layer of MSC-laden collagen type I hydrogel between the chondral and osseous layers was implemented by Lozito et al. when constructing the 3D osteochondral microtissues, described in detail in the next section²³.

6. Osteochondral tissue systems for biological research and modeling of disease

Biomimetic scaffold-bioreactor systems are increasingly used for engineering of large numbers of small-sized tissue constructs serving as *in vitro* pre-clinical models of normal tissue function and disease pathogenesis, and being used for assessing drug toxicity, efficacy and mode of action. Lozito et al. constructed an *in vitro* system with three-dimensional (3D) microtissues designed for biological studies of the osteochondral complex of the articular joint²³. The model was constructed by seeding hMSCs from bone marrow and adipose tissue aspirates into photo-stereolithographically fabricated biomaterial scaffolds with defined internal architectures.

3D printed, perfusion-ready cartridges were designed to fit into the wells of a 96-well culture plate, and maintain the osteochondral microtissues, each containing a cartilage/bone biphasic structure with a functional interface. All tissue components were derived from a single source of adult mesenchymal stem cells to eliminate any age/tissue-type mismatch. Refinements of the system include separate compartments with microenvironments for the synovial and osseous components, and possibility of applying mechanical loading and perturbations in culture conditions.

The consequences of mechanical injury, exposure to inflammatory cytokines¹¹⁵, and compromised bone quality on changes in the cartilage component can be evaluated using this versatile microsystem. It could potentially be further modified into a high-throughput *in vitro* platform for evaluation of the efficacy, safety, bioavailability, and toxicity outcomes for new drugs for treating joint disease²³.

6.1 Computational modeling

Computational models and simulations tools bring a new important perspective to osteochondral tissue engineering. Finite element modeling tools enable varying different parameters of the mechanical and biochemical environment of the cells seeded into the

scaffold. Simulations can be useful in determining and *in silico* testing the proper design and stiffness of the scaffold¹¹⁶, particularly of interest for the CAD/CAM fabrication¹¹⁷. Computational fluid dynamics can provide insight into the complex relationship between the hydrodynamic environment and engineered construct and enable improved bioreactor designs for 3D tissue culture¹¹⁸. Grayson et al. modeled the flow patterns to determine the relation between interstitial flow and tissue development. Mathematical modeling showed that the density and architecture of bone matrix correlated with the intensity and pattern of the interstitial flow, which was confirmed by the experimental data (**Figure 5**)¹⁰⁸.

7. Conclusion

It is now well established that the cells respond to all aspects of their environment, *in vivo* and *in vitro*, including signals generated by other cells, extracellular matrix and physical forces. Therefore, the main approach in OC tissue engineering is biomimetic in nature, as it aims to regulate the cell function and tissue formation by recapitulating some aspects of the native developmental milieu. The new generation of cell-scaffold-bioreactor systems is designed to stimulate the cells to undergo differentiation akin to native development and maturation, and thereby yield functional engineered tissues.

We discussed the current state-of-the-art approaches for optimization of each of the three components of the biomimetic system for OC tissue engineering: cells (different cell types and sources, chondrogenic and osteogenic differentiation protocols), stratified hierarchical scaffolds (biomaterials, design and gradient-based architectures, porosity and pore structure design, fabrication methods), with special emphasis on anatomically-shaped scaffolds and complementing anatomically-shaped bioreactors.

We highlighted protocols for engineering large, clinically sized pieces of human cartilage with physiologic stratification and biomechanics and their subsequent incorporation into OC grafts, as well as the protocols for bioreactor cultivation of anatomically shaped human bone grafts such as TMJ. With all advances, the treatment of osteochondral (OC) defects remains a challenge due to different healing abilities of articular cartilage and subchondral bone and complex interactions of these two tissues. In parallel, OC tissue constructs engineered using biomimetic scaffold-bioreactor systems are finding application as *in vitro* pre-clinical models for studying normal and pathological tissue function, modeling disease pathogenesis and screening of drugs.

8. Expert opinion

Current advances in the field of osteochondral (OC) engineering involve two paradigms that are being pursued in parallel: (i) engineering living osteochondral grafts for implantation, that are customized to the patient and defect being treated; (ii) engineering osteochondral tissues that can serve as models of disease or healing. In both cases, a biomimetic approach is followed, by engineering OC tissues under conditions that mimic the native environmental milieu of tissue development, regeneration and remodeling.

A major challenge is to differentially generate functional cellular phenotypes during differentiation of the same cells (mesenchymal cells from bone marrow, adipose tissue of

synovial fluid) into cartilage and bone cells, in a spatially and temporally defined fashion. Because the cellular material is genetically uniform, the cell fate needs to be modulated by environmental factors. As in the body, the cells will respond to the interactive and dynamic effects of the surrounding cells (a notable example is paracrine signaling between the vascular and stromal cells), extracellular matrix (with its immobilized and released factors), and physical signals (hydrodynamic, electrical and mechanical).

Clearly, to engineer a graded cartilage-bone tissue with the right morphology and function, in the same space and starting from the same cells, we need the scaffold-bioreactor systems capable of providing the right signals in the right place and time. With the technologies we already have, the main limitation is in the knowledge which exact signals (their levels, distributions, and timing) are needed, rather than in our ability to meet any specific design requirement. In other words, the effectiveness of our biologically inspired engineering designs is still largely determined by our understanding of the biological principles to follow. This in turn supports the need for controllable tissue models of high biological fidelity that can help us gain new insights into the biological processes and feedback these insights to refine our engineering designs.

A number of recent studies documents that the most effective tissue engineering approaches are those rooted in detailed understanding of the underlying biology. For example, all attempts to engineer mechanically functional human cartilage starting from hMSCs have failed until recently. While young animal chondrocytes were successfully used to grow cartilage constructs with properties approaching physiological range, the same protocols simply would not work with mesenchymal stem cells. Uniquely, when the early developmental step of mesenchymal condensation was included into the protocol for engineering *in vitro* cartilage from hMSCs, the resulting tissue acquired physiological stratification and the compressive and tribological properties of adult human cartilage⁴⁷.

The formation of condensed mesenchymal bodies (CMBs), of precisely determined size, and the precise timing of their fusion were critical for the *in vitro* formation of well-differentiated and mechanically functional layer of cartilage⁴⁷. Such cartilaginous layer of fused CMBs can be placed on the surface of the anatomically shaped porous bone scaffold that can be seeded by the same hMSCs, and cultured in a matching, anatomically shaped bioreactor chamber¹⁰⁹. Today we have several techniques that allow precise fabrication of patient-specific anatomically shaped scaffolds and bioreactor chambers.

After a functional cartilage layer interfaced with bone can be engineered by including an additional step normally present during skeletal development, the integrated technology for engineering human osteochondral grafts is now emerging. The path ahead brings significant challenges in realizing the potential to further improve engineered OC grafts, and incorporate some novel concepts from other fields.

The scaffold and bioreactor for OC constructs should contain two discrete compartments that enable cells in either region to be exposed to optimal stimuli. This is one of the biggest challenges in OC engineering – to biomimetically reproduce two very different environments in a controlled fashion: chondrogenic growth factors in combination with

dynamic loading for the cartilage phase, and osteogenic growth factors combined with medium perfusion for the bone phase. In a biomimetic bioreactor, the cartilage and bone phases should be grown in apposition while each is being adequately maintained and stimulated, in order to achieve osteochondral integration. Even though significant achievements have been accomplished as evidenced in the research highlighted in this review, there are still significant obstacles to engineering fully functional OC grafts, the major one being the establishment of an integrated interface between cartilage and bone²⁰. Future developments that are needed to address this unmet need include the implementation of “smart” scaffolds with advanced ability for local regulation of cell phenotypes, and dual-flow bioreactors providing two separate sets of conditions in the cartilage and bone compartments. Three additional directions of research are likely to play major roles in the coming years.

Bone is highly vascularized, and its development and function are coordinated by synergistic interactions between the bone cells and vascular cells. In fact, nascent vasculature serves as a template for bone development. Therefore, vascularized bone could be engineered by synchronizing vascular and bone development in 3D scaffolds^{108, 119}. Hierarchical aspects discussed extensively in this review are also important for bone vascularization. In addition to the necessity to provide paracrine signaling between the bone and vascular cells, it is critical to also provide larger vascular conduits that can help quickly connect the blood to the tissue and establish vascular perfusion following implantation of engineered tissue constructs. This is a formidable goal, not achieved using our current approaches.

We are now learning that the inflammatory response is a major regulator of vascularization and overall functionality of engineered tissues, through the activity of different types of macrophages and the cytokines they secrete. To this end, inert scaffolds that have been considered for many decades a “gold standard” in regenerative medicine are beginning to be replaced by a new generation of “smart” tissue engineering systems designed to actively mediate tissue survival and function¹²⁰. In the coming years, one should expect to see further implementation of tissue engineering strategies that take into consideration the inflammatory responses to engineered tissue constructs. We recently showed that scaffold vascularization can be achieved by manipulating the macrophage behavior, through scaffold designs that enable sequential release of immunomodulatory factors recruiting the waves of M1 and M2 macrophages¹²⁰. Harnessing the inflammatory signals of the host is emerging as a particularly effective path to enhancing tissue vascularization and integrative repair.

Finally, mechanical stimuli are intrinsic to the homeostasis of healthy cartilage and bone. Mechanical stress, fluid flow, cell-cell and cell-matrix interactions coordinate the development and function of most tissues, including cartilage and bone. Because the cells are sensing mechanical signals in their environment and converting mechanical signals into gene expression, protein activity and ultimately cell function, it is to expect that mechanoregulation of engineered OC grafts will continue to be a major focus of ongoing and future research.

Finally, it will be important to continue pursuing regenerative medicine applications of OC grafts in parallel with using microtissue platforms for predictive modeling of development

and disease in a high-throughput fashion. Conceivably, the knowledge gained with one system will benefit the other one, and lead to deeper understanding of the development, normal and pathological function of OC tissues, to benefit a range of areas: regenerative engineering, precision medicine and fundamental research.

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Article highlights

- Two paradigms are being pursued in parallel: (i) engineering of living OC grafts for implantation, customized to the patient and the defect being treated; (ii) engineering of OC tissues to serve as models of disease or healing
- Biomimetic OC engineering entails use of “smart” scaffolds with ability for local regulation of cell phenotypes, and dual-flow bioreactors providing two separate sets of conditions in the cartilage and bone compartments in order to mimic the native OC milieu
- Advanced protocols for engineering hierarchical OC grafts include mesenchymal condensation to engineer cartilage, and vascular component in the bone compartment
- New strategies take into consideration the inflammatory responses to engineered tissues

Figure 1. Osteochondral unit

Hierarchical structure comprises zonal organization of articular cartilage, transient region, and the underlying bone.

Figure 2. Fabrication of osteochondral grafts

A To form articular cartilage on bone substrate, CMBs were placed into a PDMS ring, a bone scaffold was inserted and pressed onto CMBs to cause CMBs to fuse and penetrate inside the scaffold pores resulting in a composite osteochondral construct. After differentiation, the cellular layer formed into cartilage and integrated with the porous scaffold. **B** CMBs and osteochondral constructs at day1 and week 5 post fusion (H&E). Histological and immunohistochemical sections of bioengineered cartilage and subchondral bone indicate appropriate matrix composition. **C** Top and side views of the thick layer of articular cartilage formed by fusing CMBs covering the whole construct surface (Scale bars: **B** 200 μm and **C** 2 mm.). **D** Silicone mold in the exact shape of the condyle was made in two pieces and CMBs were placed on the cartilage side. Anatomically shaped porous bone scaffold was placed on the other side, and the two-piece mold was press-fit. CMBs fused together and adhered to the scaffold as a thick cellular layer at the surface of decellularized bone that can also be seeded by the same bone marrow derived stem cells (BMSCs) used to form cartilage. The resulting construct is cultured in a matching, anatomically shaped bioreactor chamber. Adapted with permission from Bhumiratana et al PNAS 111(19): 6940-6945, 2014.⁴³

Figure 3. Biphasic osteochondral scaffold

A The scaffold was made by interfacing agarose and trabecular bone scaffolds. **B** Perfusion bioreactor for cultivation of biphasic scaffolds. Enlarged view shows the path of medium flow through the scaffolds and back into the reservoir. Reproduced with permission from Grayson et al *Osteoarthritis Cartilage* 18(5):714-723, 2010.⁶⁶

Figure 4. Tissue engineering of anatomically shaped bone grafts

(A-C) Scaffold preparation. (A, B) Clinical computerized tomography (CT) images were used to obtain high resolution digital data for the reconstruction of exact geometry of human TMJ condyles. (C) These data were incorporated into MasterCAM software to machine TMJ-shaped scaffolds from fully decellularized trabecular bone. (D) A photograph illustrating the complex geometry of the final scaffolds that appear markedly different in each projection. (E) The scaffolds were seeded in stirred suspension of human mesenchymal stem cells, to 3 million cells per scaffold ($\sim 1 \text{ cm}^3$ volume), precultured statically for 1 week to allow cell attachment and then the perfusion was applied for an additional 4 weeks. (F) A photograph of perfusion bioreactor used to cultivate anatomically shaped grafts *in vitro*. (G-I) Key steps in bioreactor assembly. Reproduced with permission from Grayson et al PNAS 107(8):3299-3304, 2010¹⁰⁸.

Figure 5. Bone matrix morphology correlated to the patterns of medium perfusion flow (A and B) Computational models of medium flow through temporomandibular joint (TMJ) constructs during bioreactor cultivation. (A) Color-coded velocity vectors indicate the magnitude and direction of flow through the entire construct based on experimentally measured parameters. (B) Construct is digitally sectioned, and the color-coded contours are used to indicate the magnitude of flow in the inner regions. Reproduced with permission from Grayson et al PNAS 107(8):3299-3304, 2010¹⁰⁸.