PERSPECTIVE

Bioprinting of osteochondral tissues: A perspective on current gaps and future trends

Pallab Datta[1], Aman Dhawan[2], Yin Yu[3,4], Dan Hayes[5,6], Hemanth Gudapati[5,7] and Ibrahim T. Ozbolat[5,6,7,8*]

[1] Centre for Healthcare Science and Technology, Indian Institute of Engineering Science and Technology Shibpur, Howrah, West Bengal 711103, India
[2] Orthopedics and Rehabilitation, Penn State University, Hershey, PA 17033, USA
[3] Department of Surgery, Harvard Medical School, Harvard University, Cambridge, MA 02138, USA
[4] The Center for Engineering in Medicine, Massachusetts General Hospital, Boston, MA 02114, USA
[5] The Huck Institutes of the Life Sciences, Penn State University, University Park, PA 16802, USA
[6] Biomedical Engineering, Penn State University, University Park, PA 16802, USA
[7] Engineering Science and Mechanics Department, Penn State University, University Park, PA 16802, USA
[8] Materials Research Institute, Penn State University, University Park, PA 16802, USA

Abstract: Osteochondral tissue regeneration has remained a critical challenge in orthopaedic surgery, especially due to complications of arthritic degeneration arising out of mechanical dislocations of joints. The common gold standard of autografting has several limitations in presenting tissue engineering strategies to solve the unmet clinical need. However, due to the complexity of joint anatomy, and tissue heterogeneity at the interface, the conventional tissue engineering strategies have certain limitations. The advent of bioprinting has now provided new opportunities for osteochondral tissue engineering. Bioprinting can uniquely mimic the heterogeneous cellular composition and anisotropic extra-cellular matrix (ECM) organization, while allowing for targeted gene delivery to achieve heterotypic differentiation. In this perspective, we discuss the current advances made towards bioprinting of composite osteochondral tissues and present an account of challenges—in terms of tissue integration, long-term survival, and mechanical strength at the time of implantation—required to be addressed for effective clinical translation of bioprinted tissues. Finally, we highlight some of the future trends related to osteochondral bioprinting with the hope of in-clinical translation.

Keywords: bioprinting; osteochondral injuries; zonal anisotropy; bioink; tissue engineering

*Correspondence to: Ibrahim T. Ozbolat, The Huck Institutes of the Life Sciences, Penn State University, University Park, PA 16802, USA; Email: ito1@psu.edu

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1. Introduction: Current Status of Osteochondral Tissue Bioprinting

Damage to the articular surface in the form of localized cartilage erosion is usually observed in relation to joint degenerative disease(s) such as osteoarthritis (OA) or trauma[1]. Post-traumatic OA is particularly significant, since it affects a demographic that is considered too young for joint replacements. As such, OA is one of the major chronic conditions plaguing our society, causing considerable pain and debilitation, affecting an estimated 21 million people with an economic burden of $89.1 billion annually in the US[2–4]. Surgical methodologies aimed at cartilage healing, such as microfracture, grafting and autologous chondrocyte implantation, are often complicated, costly or yield unsatisfactory results in the long-term, especially in the elderly population, eventually needing joint replacement to restore normal function[5–8]. To this end, regenerative medicine that aims to repair, regenerate and improve functionality of injured/diseased tissues holds great potential in osteochondral therapy, which involves both the cartilage and bone and has generated significant interest. However, challenges still exist in achieving this goal of engineering tissue structures that can closely mimic the

native osteochondral tissues individually as well as the interface region[9]. The heterogeneous and anisotropic cartilage is generally considered as a layered structure of "zones" that possess mechanical properties reflecting each zone's compositional and architectural make-up[10]. The layered arrangement of chondrocytes and bone cells are unique feature of the osteochondral tissue as shown in Figure 1, which is difficult to recapitulate using current regenerative approaches. Although various scaffolding approaches and materials have been used to date[5], successful regeneration of large articular cartilage with native-like biological, mechanical and structural characteristics is still a challenge. Similarly, individual challenges also remain for bone-tissue engineering, making the regenerative strategies for composite tissue even more challenging[11,12].

1.1 Drawbacks of Current Tissue Engineering Approaches for Osteochondral Regeneration

Articular cartilage repair is a highly challenging clinical problem for orthopaedic surgeons[13]. Adult articular cartilage has limited intrinsic repair capacity due to its avascular nature[14]. Even a minor focal lesion can cause progressive cartilage damage, affecting the whole articulating joint and increasing the risk of developing OA. Traditional cartilage repair techniques focus on pain relief as well as restoring tissue function[5]. Osteochondral injury, depending upon the size and chronicity, may be more of a challenge to treat. Smaller acute lesions (less than 2 cm^2) are filled with type-I collagen fibrocartilage, which has been proven biomechanically and histologically inferior to native hyaline cartilage[15]. Larger lesions (greater than 2 cm^2) require addressing the underlying subchondral bone in addition to the articular cartilage. There are five clinically available treatment options for osteochondral restoration. These include: 1) osteochondral autograft, 2) osteochondral allograft, 3) impaction bone grafting, 4) Autologous chondrocyte implantation (ACI) "Sandwich Technique", and 5) biphasic scaffolds[16–26]. These techniques, along with their strengths and limitations, are summarized in Table 1.

Currently, the most commonly utilized restorative option with the most data is osteochondral allograft, which combines viable donor subchondral bone and overlying hyaline articular cartilage[17,18]. This may be performed utilizing a shell or dowel technique incorporating differing amounts of subchondral bone, and can be titrated to the specific clinical situation and needs[18,24]. This graft provides living osteoblasts and osteocytes, as well as chondroblasts and chondrocytes, along with well-organized extracellular matrix to the defect without the donor site morbidity of autograft osteochondral plugs (mosaicplasty). While commonly utilized and demonstrated good short-term success, long-term studies demonstrate only 66% graft survival

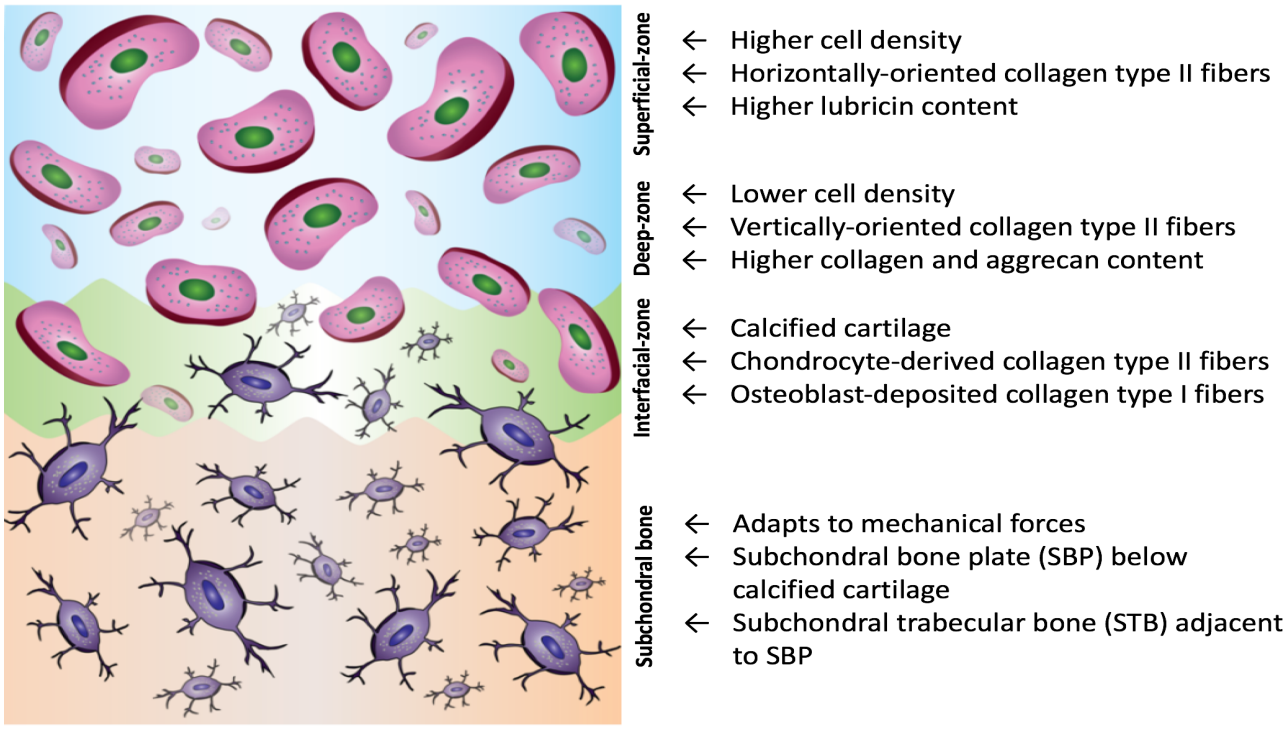

Figure 1. A schematic showing the osteochondral tissue with stratified layers and their characteristics

Table 1. Clinical options for osteochondral restoration

Technique	Pros	Cons	References
Osteochondral osteoarticular allograft	• Restoration of architecturally correct hyaline cartilage and bone with viable bone and cartilage cells • Excellent short-term patient reported outcomes and survivability	• Disease transmission and immunogenicity • Availability • Contouring challenges in patellofemoral joint • Short-term clinical results not sustained through long-term follow-up	[16–18,24,27,95]
Osteochondral autograft (mosaicplasty)	• Restoration of architecturally correct hyaline cartilage and bone with viable bone and cartilage cells • No disease transmission or immunogenicity concerns	• Donor site morbidity • Contouring challenges in all locations	[15–18]
Impaction bone grafting (allo- or autograft)	• Can be performed with auto- or allograft • Inexpensive	• Limited data • No biologic restoration of articular surface	[23]
ACI "Sandwich Technique"	• Restoration of architecturally correct hyaline cartilage and bone with viable bone and cartilage cells • Contouring can be modified to all compartments of the knee	• Limited data • Expensive	[96,97]
Biphasic scaffolds	• Directed bone and cartilage restoration using bio-directive matrix • Relatively cheap	• Mixed clinical results with even short-term follow-up • Breakdown products from bioresorbable materials may be chondrotoxic and detrimental to the surrounding cartilage and bone.	[25,26]

at 20 years[27]. Osteochondral allograft is useful for lesions of the femoral condyles as well as to a lesser extent the tibial plateau[24]. Its use in the patellofemoral compartment is limited because of surface contour matching and complexity. Osteochondral allograft, because of its viability, is particularly susceptible to both donor rejection and incorporation issues, as well as disease transmission[16,18,24]. In addition, because it is the most commonly utilized restorative option, availability is becoming more challenging, often with wait times of over a year, during which time continued pain, functional limitations, and lost work and wages may be experienced. Failure of use of osteochondral allografts is associated with age at time of primary allograft, number of previous surgeries, size of defects, and bicondylar involvement. Patients who are of age 30 years or older at the time of osteochondral implantation have a 3.5 times greater risk of failure as compared to younger patients[24,27].

A cellular scaffolds (more practical in clinical settings) and cellularized scaffolds (loaded with cells, such as chondrocytes[28], mesenchymal stem cells (MSCs)[29,30] and human-induced pluripotent stem cells (hiPSCs)[31,32]), give structural support to recruited or loaded cells for their proliferation and differentiation, and regeneration of extra-cellular matrix (ECM) until the scaffold degrades. For acellular scaffolds, biochemical cues are usually applied, including stromal cell-derived factor alpha 1 (SDF-1α)[33], which helps the recruitment of progenitor cells, and transforming growth factor β (TGF-β) family (*i.e.*, TGF-β1 and TGF-β3[34]), which promotes the differentiation of recruited progenitor cells into chondrocytes and enhances biochemical composition and functional properties of the regenerated tissue. The major challenge with the scaffold-based techniques is the degradation of scaffold matrix and its complications such as toxic products and abrupt changes in mechanical properties after implantation. Most importantly, cells in scaffolds are limited in their interactions and signaling while they are confined in gel matrix[35]. This is particularly important in differentiation of cells, as well as the mechanotransductive signaling between cells that facilitate successful regeneration of anisotropic tissues. Cell-free graft techniques, which use biocompatible and degradable materials as a scaffold to support endogenous tissue regeneration, show promise in animal models but have yet to find clinical success[36–38]. Cell-seeded, biphasic scaffolds may serve as an integrated solution to recapitulate the osteochondral interface and underlying bone[37,39], but despite the success in pre-clinical studies, only three biphasic osteochondral scaffolds have extensive clinical application[40]. The use of these biphasic systems has resulted in mixed outcomes with frequent failures to restore subchondral bone and long recovery periods[40–42].

Instead of using exogenous biomaterials, scaffold-free

approaches have been recently used, where chondrocyte spheroids (also known as chondrospheres[43]) self-assemble into articular cartilage when implanted into the lesion, and has been under investigation in a phase-III controlled clinical trial in Europe[44]. Chondrospheres have similar properties to native cartilage and can be engineered by altering their cellular density, self-assembly and culture condition.

1.2 Three-dimensional (3D) Printing for Osteochondral Defect Healing

Attempts to create bi-layered grafts for osteochondral tissue regeneration have been further boosted by the development of three-dimensional (3D) printing technology. Initially, 3D printing was used in conjunction with conventional scaffold fabrication techniques, such as particulate leaching, to obtain bi-layered structures. In most such cases, polymeric scaffolds have been selected to mimic the cartilage tissue, whereas a ceramic phase is usually chosen to represent the subchondral bone. For example, hydroxyapatite has been printed with a porous polylactide (PLA) scaffold to mimic the osteochondral tissue composition and *in vivo* results exhibited osteogenic and chondrogenic markers in both respective layers[45]. Similarly, stereolithography process has been used to fabricate osteochondral constructs with polyethylene glycol and beta (β)-tricalcium phosphate, which showed encouraging results in a year-long follow-up study in a rabbit critical-size defect model[46]. Using fused deposition modeling, Cao *et al*. fabricated a honey-comb-like PCL scaffold with 0°/60°/120° lay-down pattern to create anisotropic structures[47]. Using 3D printing technology, tissue constructs with porosity gradient with embedded nanomaterials have been demonstrated for osteochondral healing[48,49]. Furthermore, MSCs and chondrocytes cultured on such scaffolds showed different tissue morphologies over time[48]. Similar experiments using fibrin glue to mimic the cartilage tissue have also been reported[50,51]. 3D printing using selective laser sintering is also a facile technique to create gradient porosity[52,53]. Though 3D printing techniques allow for creating different mechanical and porosity properties, inferior cell–cell interactions and inhomogeneous cell growth and differentiation amongst the scaffold remain the barriers for effective clinical translation.

1.3 Bioprinting for Osteochondral Engineering

Bioprinting is a process by which living cells and biomaterials can be deposited precisely in a layer-by-layer manner as per a prescribed computer-aided design for the fabrication of engineered tissue constructs[54]. Based upon the mechanism of deposition, bioprinting can be defined in three broad categories—extrusion-based bioprinting (EBB), droplet-based bioprinting (DBB), and laser-based bioprinting (LBB)—the detailed mechanisms of which are available at several sources[55,56]. The bioprinting techniques offer several advantages for engineering of osteochondral tissue constructs. Bioprinting allows for precise mimicking of the native heterogeneous, anisotropic tissue architectures. Most bioprinting techniques presently have the capability to process several different types of cells and biomaterials, rendering unique potentiality to tune structural and mechanical properties as per the requirement of specific tissue-type. In the case of osteochondral tissue, wherein the mechanical and compositional requirements are different for cartilage and bone tissues, bioprinting can thus be advantageous. Moreover, the ability to precisely control the patterning of cells and biological materials enables the fabrication of zonal variations seen in osteochondral tissue. Interestingly, all the processing and fabrication of labile biological materials, such as genes, growth factors and cells, through bioprinting can be achieved under physiologically ambient conditions. It has been thus observed that bioprinted constructs allow for precise facilitation of cell–cell interactions, which is critical to fabricate a composite tissue[57–61]. Thus, bioprinting has attracted the attention of researchers working with the quest to devise improved solutions for osteochondral healing.

One of the early osteochondral tissue bioprinting efforts was attempted with EBB of two different cell types: mesenchymal stem cells with osteoinductive calcium phosphate particles, and chondrocytes on two sides of an alginate mesh scaffold[62]. After approximately three weeks in culture as well as *in vivo* experimentation, functional markers and ECM characteristics of both osteogenic and chondrogenic differentiation were observed indicating the formation of interfacial composite tissue. Later research has shown that bioprinting of cells with an appropriate hydrogel can be used to direct differentiation into desired tissue. In these studies, collagen type-I or polycaprolactone (PCL) was found to be suitable for bone tissue formation, and hyaluronic acid or alginate was suitable for cartilage tissue formation[63]. As depicted in Figure 2A**,** a separate study has shown that droplet-based bioprinting can be effectively used to obtain composite tissue, where human mesenchymal stem cells (hMSCs) were bioprinted on patterned bone morphogenetic protein-2 (BMP-2) committed to osteoblast formation, while MSCs were bioprinted on patterned TGF-β1 committed to chondrocyte differentiation[64]. Using a Bioscaffolder®, a potential method to generate osteochondral models of clinically-relevant sizes using poly(lactic acid) microcarriers has been developed[65]. This study explored the fabrication of a bilayered graft in which cartilage

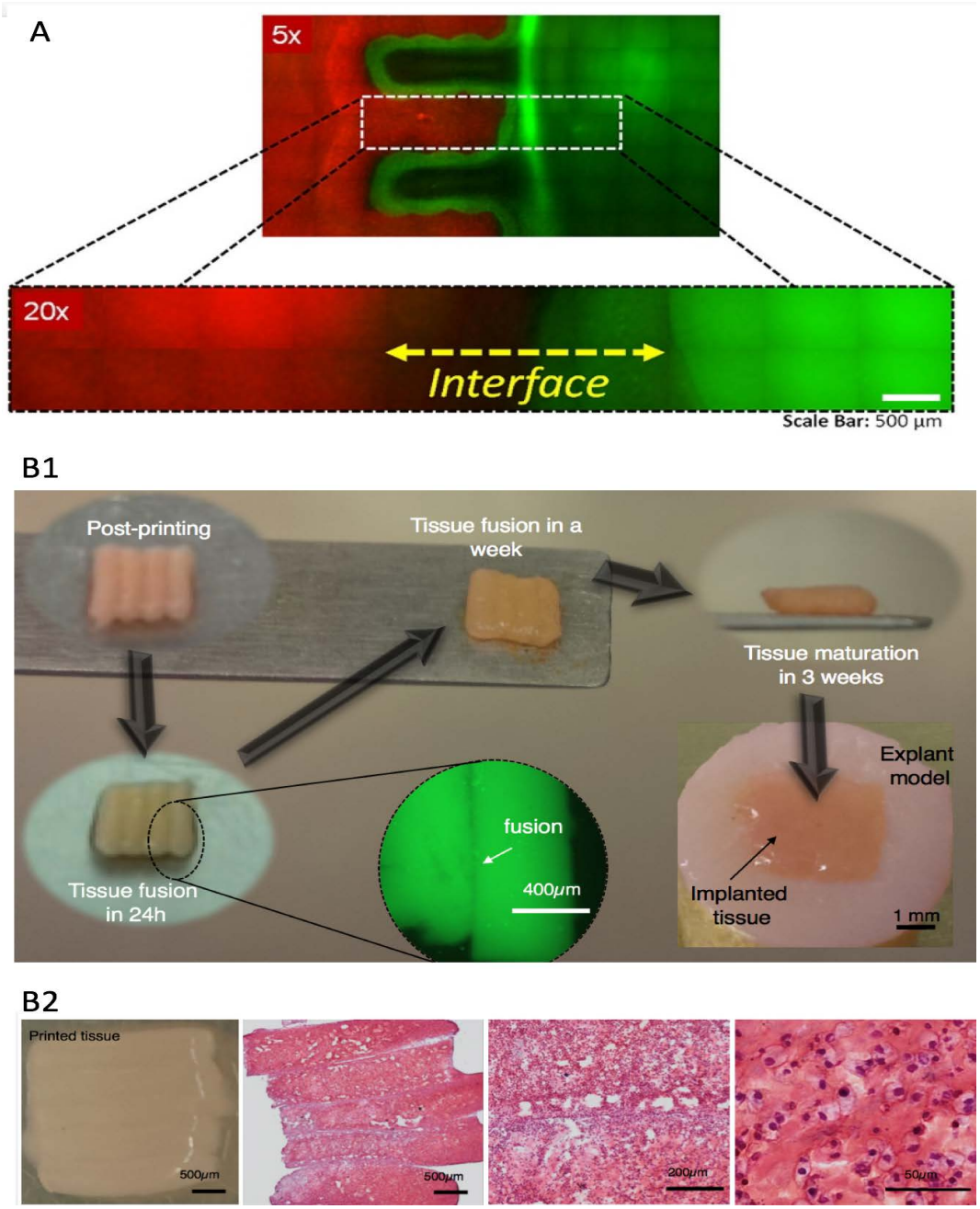

Figure 2. Successful fabrication of tissue constructs by bioprinting: (**A**) Fibrocartilage transition region of around 1–2 mm in length, obtained by bioprinting of encapsulated hMSCs with TGF-β1 (red color zone) and BMP-2 (green color zone) patterns (reproduced/adapted from Gurkan *et al.*[64]), and (**B1**) bioprinting cartilage tissue strands facilitated their rapid fusion and maturation into (**B2**) a single patch of articular cartilage demonstrating proteoglycan formation and integration of interface regions (reproduced/adapted from Yu *et al.*[94])

region was printed with gelatin methacrylate-gellan gum (GelMA-GG) and the bone region was represented by GelMA-GG with encapsulated microcarriers (MCs). Poly(lactic-*co*-glycolic acid)–poly(ethylene glycol) microspheres with controlled release of BMP-2 has been demonstrated with maintenance of cell viabilities post-bioprinting to engineer osteochondral tissue constructs[66].

2. Towards Mimicking the Heterogeneity and Anisotropy

Articular cartilage is the lining on articulating surfaces of diarthrodial joints and it functions as a shock absorber to distribute the load from weight and daily activities[67]. Articular cartilage is responsible for resisting compressive stress and enables a proper distribution

of mechanical loading on the subchondral bone. Another important function of articular cartilage is lubrication of the joint. Lubricants, such as proteoglycan 4, reduce friction between contacting surfaces, thus minimizing wear and tear of the joint[68]. One of the hallmarks of the osteochondral interface is the zonal variations in the structure, property of articular cartilage and the subchondral bone, which makes the design of tissue engineering scaffolds challenging. Chondrocytes organize extracellular matrix in to unique and highly specialized tissue. Articular cartilage can be divided into the superficial zone, transitional zone and middle (radial) or deep zone, and calcified cartilage zone. It varies in composition of primary constituents *viz.* water, collagen, proteoglycans, chondrocytes and some other minor proteins. The superficial-zone takes up to 20% of the total cartilage thickness and cells in that zone secrete lubricants. It contains densely packed collagen fibers in parallel to the articulating surface to resist shear stress and to protect the joint. The deeper zones, including middle-zone, deep-zone and calcified zone, are relatively less in cell density and have thicker collagen bundles, which are perpendicular to the articulating surface. Deeper zones help articular cartilage to resist compression force. The subchondral bone, on the part, is composed of concentric lamellar layers around the osteons and flat layers representing new bone formation. The peripheral bone is largely avascular, while the endosteal bone abuts directly on calcified cartilage[69].

The unique anisotropic arrangement is formed due to the external loads over time, which is transmitted through the matrix of the tissue and converted into a biochemical signal, alerting cells to either produce more or catabolize existing ECM[5]. Scaffold-based tissue engineering approaches interrupt this transmission as the scaffold material confines the cells and shields cells from this mechanotransductive signaling cascade[70]. Thus, novel scaffold-free tissue engineering approaches are needed to help preserve the natural balance between external mechanical loading and the maintaining of zonal microenvironments for chondrocytes to adapt and regulate their biosynthetic activities in order to produce zonally-stratified characteristics of cartilage. Moreover, at the osteochondral interface, chondrocytes from the calcified cartilage zone and cells from subchondral bone differ in their differentiation status and metabolic activities, making any tissue engineering strategy to recapitulate this interface very challenging. The heterotypic cell-specific differentiation should not compromise the mechanical integrity of the interface. In general, the compressive modulus of articular cartilage increases from superficial layer (0.079 MPa) to 2.10 MPa in the deepest zone, while the tensile modulus varies in inverse direction, reducing from 25 MPa (superficial zone) to 15 MPa in deep zone[71]. On the other hand, the modulus values of subchondral bone range higher than values obtained with biofabricated constructs, which mostly lie in 30–3,000 kPa range[72,73]. In this aspect, though hydrogel materials have been the preferred choice for cartilage bioprinting, the mechanical properties of the subchondral bone demand a more robust support structure such as PCL.

To mimic the zonal compositions, a Fab@Home 3D printer has been shown to effectively deposit PLGA–PEG microspheres co-printed with alginate-cell suspension in multilayered structures[66]. Present bioprinting capabilities are adequate to obtain scaffolds with mimetic osteochondral mechanical, biochemical and porosity gradients. Custom-developed 3D bioprinters have been used to create multilayered osteochondral tissue constructs by bioprinting human turbinate mesenchymal stem cells (htMSC) on a slowly degrading PCL frame[74,75]. Using this approach, htMSCs with atelocollagen and recombinant human bone morphogenetic protein (rhBMPs) have been bioprinted over the PCL layer, creating a layer with a thickness of 4 mm to mimic the subchondral bone tissue. This was followed by bioprinting of htMSC-HA-TGF-β at 1-mm thickness on the subchondral bone structure to mimic cartilage tissue. The constructs showed promising results in the repair of rabbit knee joints. Recently, an EBB platform has been combined with a multi-nozzle electrospinning technique to fabricate gradient constructs with differential release rates of gentamycin sulfate (GS) and desferoxamine (DFO), which can be extended to co-print cells[76].

Despite some success of 3D bioprinting as a tool for osteochondral tissue regeneration, developing an integrated construct closely mimicking the heterogeneity and anisotropy of articular cartilage, subchondral bone and the soft–hard interface remains a critical challenge. Solving this challenge plays a crucial role in improving the osteochondral tissue regeneration process and graft integration with host tissue[77]. Many of the limitations for traditional osteochondral tissue engineering approaches can be attributed to the inability of precise spatiotemporal and temporal control of biomechanical and biochemical cues for direct cell migration, differentiation and cell–cell interaction. 3D bioprinting-based approaches to engineer osteochondral tissue can provide precise spatial control of bioactive compounds and biomaterials to mimic the gradients of biologic and mechanical signals along the osteochondral axis. For example, growth factors for chondrogenesis and osteogenesis, and plasmid DNA encoding osteo- and chondrogenic genes and siRNA modulators of differentiation, can be integrated into "bioinks" made of biomaterials with different mechanical properties for cartilage and bone tissue, respectively. Several miRNAs,

miR-26a, -148b, -27a, and -489, have shown to regulate osteogenesis in MSCs[78–80]. Of these compounds, miR-148b has been shown to induce *de novo* osteogenesis in bone marrow-derived MSC and ASC and has been demonstrated to enhance progenitor osteogenesis and bone repair both *in vitro* and *in vivo*[79,81–83]. Several miRNAs such as miR-146a, miR-9, miR-29a and miR-140 play a role in the regulation of chondrogenesis[84–89]. In addition, different drug delivery systems can be incorporated into "bioinks" and be deposited in regions that require controlled release of cell-signaling molecules[90]. For example, at the osteochondral interface, chemokine-guided migration of endogenous cells is desired to provide subchondral bone integration followed by cell differentiation[33]. By modulating bioprinting parameters, the release of chemokines and cytokine signals promoting cell migration and differentiation can be controlled in a sequential manner providing for precise spatiotemporal control of osteochondral tissue development.

3. Challenges in Clinical Translation of Bioprinted Osteochondral Tissues

Challenges to clinical translation include both clinical and administrative/translational[91]. Clinically, the bioprinted tissue will need to incorporate with the surrounding host chondral surfaces and host subchondral bone. If allogeneic, this will be expected to have similar potential incorporation challenges as viable allograft osteoarticular allografts. Autograft osteochondral composites will need tissue harvest, cell expansion, printing and the maturation of the fabricated construct, making one-stage printing *in-situ* challenging. The composite printed grafts themselves will need sufficient biomechanical strength at implantation to sustain joint motion and immediate rehabilitation to avoid iatrogenic stiffness and pain. This will be more of a challenge with larger animal models as well as clinical translation. To help reconcile, customized bioreactors will need to be designed for cultivating bioprinted grafts to enhance their biological and mechanical time-zero properties. Post-operative rehabilitation will have to account for initial time-zero strength with limited weight bearing for a small period of time. In addition, the inflammatory milieu in the synovium with osteoarthritis will have to be addressed so as to not create an unfavorable environment for the bioprinted graft. This may require biological treatment directly to the synovium or possibly incorporated into the graft. Administratively, like many chondral and osteochondral restorative options, bioprinting of composite osteochondral tissues will need to demonstrate cost-efficacy. As many of the components (and equipment needed) to graft fabrication may be very expensive, cost prohibition may be an issue. This, like all technologies, will likely become more cost-effective over time, with more efficiency in production and equipment manufacturing. Last, but often most challenging, will be the often daunting regulatory hurdles for clinical translation from cartilage basic science[92,93]. This can prove costly and time-consuming, and many technologies and developers have floundered when attempting to jump through the many hoops of the regulatory process.

4. Future Perspectives

Although research in 3D bioprinting is expanding at a rapid rate, it is essential that tissue-specific development roadmaps are adopted by independent groups to delivery clinically-relevant constructs. With particular regards to osteochondral tissue, it is critical that bioprinting is capable of manipulating both soft- as well as hard-matrix materials. In this context, it may be a viable concept to explore scaffold-free bioprinting for cartilage tissue and a mechanically strong support matrix for bone tissue. Already, scaffold-free bioprinting has recently shown promise towards cartilage differentiation for osteochondral healing as shown in Figures 2(B1) and (B2)[94]. If these can be combined effectively with PCL biofabrication, a viable solution can be designed. Secondly, with respect to the tissue heterogeneity, advances in designing of constructs with gradient porosity are essential. The gradient scaffolds should also be capable of delivering growth factors/genes with precise spatiotemporal control to achieve functional constructs in required time frames.

Conflict of Interest and Funding

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