From Intricate to Integrated: Biofabrication of Articulating Joints

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ABSTRACT: Articulating joints owe their function to the specialized architecture and the complex interplay between multiple tissues including cartilage, bone and synovium. Especially the cartilage component has limited self-healing capacity and damage often leads to the onset of osteoarthritis, eventually resulting in failure of the joint as an organ. Although in its infancy, biofabrication has emerged as a promising technology to reproduce the intricate organization of the joint, thus enabling the introduction of novel surgical treatments, regenerative therapies, and new sets of tools to enhance our understanding of joint physiology and pathology. Herein, we address the current challenges to recapitulate the complexity of articulating joints and how biofabrication could overcome them. The combination of multiple materials, biological cues and cells in a layer-by-layer fashion, can assist in reproducing both the zonal organization of cartilage and the gradual transition from resilient cartilage toward the subchondral bone in biofabricated osteochondral grafts. In this way, optimal integration of engineered constructs with the natural surrounding tissues can be obtained. Mechanical characteristics, including the smoothness and low friction that are hallmarks of the articular surface, can be tuned with multi-head or hybrid printers by controlling the spatial patterning of printed structures. Moreover, biofabrication can use digital medical images as blueprints for printing patient-specific implants. Finally, the current rapid advances in biofabrication hold significant potential for developing joint-on-a-chip models for personalized medicine and drug testing or even for the creation of implants that may be used to treat larger parts of the articulating joint. © 2017 The Authors. *Journal of Orthopaedic Research* Published by Wiley Periodicals, Inc. on behalf of the Orthopaedic Research Society. J Orthop Res 35:2089–2097, 2017.

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Worldwide spending on three-dimensional (3D) printing is expected to surpass $35 billion in 2020[1] and the technology holds promise for significant breakthroughs in medicine.[2] Like in other medical fields, also in orthopaedics additive manufacturing (AM) is driving a shift toward mass personalization, as personal scans can be converted into computer aided design (CAD) files, which are then used to design perfectly fitting surgical guides[3] or other tools.[4] The technology also allows for the generation of personalized external prostheses, which are, for example, mirrored from the healthy other limb.[5] In addition, personalized implants can be designed and printed on demand for complex revisions of endoprostheses, in trauma cases[6] or for reconstruction after tumor resection surgery.[7] Although these developments will impact current treatment of joint damage, these approaches rely on synthetic and metallic materials that lack any biologically adaptive properties and cannot remodel with host tissues. The emerging field of biofabrication addresses this issue in tissue engineering and regenerative medicine, as it uses cells and bioactive materials in its fabrication process. Biofabrication is defined as "the automated generation of biologically functional products with structural organisation from living cells, bioactive molecules, biomaterials, cell aggregates such as microtissues, or hybrid cell-material constructs, through bioprinting or bioassembly and subsequent tissue maturation processes."[8] Biofabrication, therefore, potentially can deliver a biologically responsive implant that could address some important challenges that are currently faced in the treatment of articulating joints.

Anatomically sized implants with a patient-specific shape could be provided by biofabrication following the same lines as AM. Such a personalized anatomical shape will secure smooth seamless transition between graft and host, contributing to an appropriate fit. This will avoid unnecessary wear and ensure mechanical stability of the joint. Nevertheless, there are more aspects to articular regeneration than just joint geometry.

The layered structure of cartilage is essential to ensure proper physiologic and mechanical functioning, and assuring a firm integration between all these layers is crucial for producing an implant stable enough to withstand the mechanical forces that are generated in a joint. Implants should preferably come close to the mechanical characteristics of native tissue, especially in those situations where tissue-engineered and original tissues sit close together.[9] Clearly, proper fixation of an implant is a prerequisite for effective integration between both cartilage and

Abbreviations: 3D, three-dimensional; AM, additive manufacturing; β-GP, beta-glycerophosphate; CAD, computer aided design; ECM, extracellular matrix; GAG, glycosaminoglycan; IGF-1, insulin-like growth factor-1; MEW, melt electrospinning writing; MSCs, mesenchymal stem cells; PCL, Polycaprolactone; TGF-β1, transforming growth factor β1

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bone from host and implant. Likewise, an implant would fail if the integration between the cartilage and bone compartment, which in native tissue is provided by the calcified cartilage, is not sufficient.

Biofabrication can potentially deal with the above-mentioned requirements using cells, multiple materials and biochemical compounds. As cells and bioactive molecules are key factors in the regenerative response, the ability of biofabrication techniques to orchestrate spatial concentrations of bioactive factors and/or cells, either through direct placement and/or by controlling the architecture of the implant[10–12] makes it a valuable tool in tissue engineering. This review discusses the current and future assets and opportunities of biofabrication to address challenges in treatment strategies for cartilage repair, particularly for replacing larger parts of the joint (Fig. 1).

Mimicking the Layered Structure of Native Tissue

The relatively simple appearance of articular cartilage, which is avascular, aneural, and contains only one cell-type,[14] is deceptive and attempts at cartilage repair using implants with relatively homogenous structures have hitherto not succeeded in creating clinically successful products able to regenerate the articular surface.

The intricate mechanical characteristics of articular cartilage are dictated by the complex zonal structure of the tissue,[15–18] consisting of three layers with distinct composition and architecture: the shear and tension resistant superficial zone; the intermediate middle zone; and the deeper zone with its high compressive stiffness.[16,19] From the articulating surface toward the bone, these layers show a decrease in cell density and water content combined with an increasing glycosaminoglycan (GAG) and collagen content, while collagen fibril alignment gradually pivots[15–18] according to the arcade model described by Benninghoff.[20] Together, these depth-dependent differences create a structure with unique gradually changing mechanical properties, dictating the variance in protein secretion and extracellular matrix (ECM) composition.[15,21,22]A major challenge is to induce zone specific matrix production in engineered tissues. This can be done by orchestrating the spatial and temporal presentation of multiple growth factors and mechanical cues. For instance, the activity of transforming growth factor β3 (TGF-β3) acted synergistically with oscillatory application of hydrostatic pressure to enhance cartilage production in human adipose-derived stem cells.[23] This was confirmed for human mesenchymal stem cells (MSCs) and multi axial loading even appeared to activate latent TGF-β1 incorporated in the medium.[24] The combination of insulin-like growth factor-1 (IGF-1) with TGF-β1, in the middle zone of a construct, promoted chondrogenic differentiation of human MSCs.[21,25] Further, TGF-β1 and BMP-7 enhanced expression of superficial zone markers and TGF-β1 combined with hydroxyapatite led to expression of calcified zone markers.[25] Additionally, differences in zone-specific fiber or scaffold orientation, created by conventional techniques, influenced the expression of zonal markers[26,27] and either osteogenic or cartilaginous differentiation of chondrocytes could be induced by variance in matrix stiffness.[21] Such zonal complexity and combination of factors can be effectuated by biofabrication, which has the ability of tuning the micro architecture by depositing multiple materials to create gradients or reinforcing fibers in multiple directions, providing the possibility of steering local differences in the cartilage that will eventually be produced. It has further been demonstrated that direction of both cell alignment and collagen formation can follow the geometry of deposited polymer strands when subjected to an adequate strand spacing ($<200\,\mu m$)[28] or to aligned nanofibers.[29] It remains challenging, however, to replicate the orientation of the collagen fibers, as the resolution of the available printing processes is still below the required resolution to mimic the Benninghoff arcades.

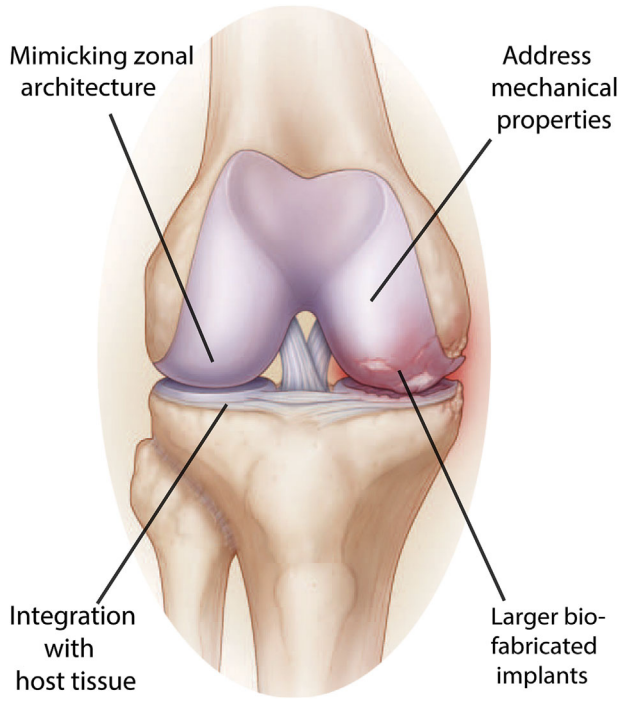

Figure 1. Challenges in biofabrication of articulating joints. Reproduced and adapted with permission from the NEJM group.[13]

Zonally organized constructs that were subsequently seeded with cells have been produced by AM.[22,30,31] A gradient in pore-size of printed polymer scaffolds was shown to alter cell distribution, although no influence on tissue composition was observed.[22] Seeding chondrocytes from specific zones on 3D printed zonal polymer scaffolds induced the formation of abundant cartilage-like tissue, yet chondrocytes lost their zone-specific characteristics.[31]

Biofabrication can incorporate cells in these fabrication processes and has already been used to create zonally organized composites (Fig. 2).[32–34] For

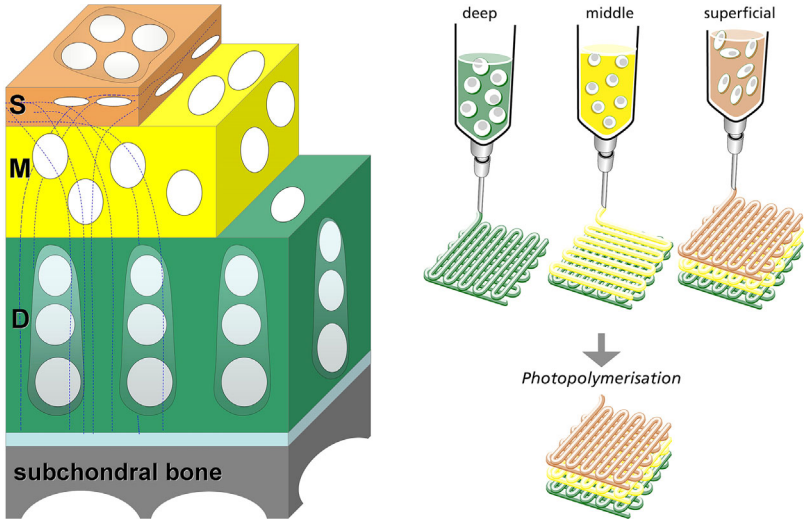

Figure 2. Schematic representation of the zonal organization of articular cartilage, showing how cell morphology and collagen fiber orientation vary across the thickness of the tissue. Multiple zone-specific bioinks could be used in a biofabrication set-up to replicate the zonal chondrocytes and ECM phenotype via printing in a layer-by-layer fashion. Reproduced with permission from John Wiley&Sons.[16]

example, a cell density gradient was bioprinted with a piston-driven depositional print head on a robotic arm. This promoted formation of a gradient distribution of ECM, which was correlated to the cell density.[32] Also, a modified thermal inkjet printer was used in combination with simultaneous photo-polymerization to deposit and crosslink a bioink. This produced an even cell distribution, however, modifying the time between photo-polymerization can create a zonal distribution of cells due their gravitation-driven movement.[33,34]

Chondrocytes from specific zones have been used and were shown to respond differently to co-culture systems,[35] mechanical stimuli,[36] and biochemical compounds.[37] Nevertheless, dedifferentiation and loss of zone-specific characteristics are major challenges.[17,31] Besides, zonal harvesting techniques have not been optimized, are time consuming and chondrocyte yield is generally low.[15,38] Ultimately, the etiology of the zonal differences has not yet been sorted out. It could even be questioned if zone-specific or different types of chondrocytes exist at all, because chondrocytes could also express zone specific markers due to their spatial position and consequentially distinct mechanical stimuli they are subjected to. In our opinion, the use of chondrocytes from a specific zone seems like an overcomplicated strategy that is probably of no added value in the clinic.[15]

While much knowledge has been gained regarding the response of chondrocytes to different stimuli, this knowledge is unfortunately not enough to generate a biologically functional graft for in vivo application that is able to create the desired organizational structure. To achieve this ambitious goal, multiple strategies will have to be combined.[39,40] The layer-by-layer fashion in which biofabrication assembles its products, combined with the ability to incorporate different growth factors,[41,42] vary cell densities and tailor fiber orientation, seems to meet all necessary requirements to create such complex biologically functional osteochondral implants.

Mechanical Properties of the Implant Approaching Those of Native Tissue

Articular cartilage is a biomechanical tissue *par excellence*, of which the properties and functions are largely dictated by its composition and structure.[43,44] Basically, the role of intact hyaline cartilage is to function as a cushion between two opposing rigid bones in articulating joints, by distributing load and consequently decreasing stress at the contact point. In addition, cartilage ensures that movements occur under minimal friction and wear.[43] Restoration of biomechanical function is one of the crucial requirements of any attempt to revive joint function for a long-term period, as an implant must withstand the substantial loading stresses associated with locomotion and sometimes even athletic activity.

Biofabrication generates cell-laden constructs by means of hydrogel-based bioinks. Hydrogels are very suitable for mimicking the native ECM as they provide a highly hydrated environment favorable for cells. For optimal printability, a hydrogel has to display shear thinning behavior, rapid gelation and little or no extrudate swell.[45] Several strategies have been used to improve rheological properties of hydrogels, such as the incorporation of additives tuning viscosity, yield stress and gelation kinetics, resulting in an improved printability and shape-fidelity after strand deposition.[46–48] However, the design of hydrogels for bioprinting is challenging as the rheological properties also have to allow for biological activity of the cells.

Importantly, hydrogels are limited by low compressive stiffness,[49,50] regardless of the crosslinking process they can be subjected to. Constructs simply composed of a hydrogel will not be appropriate for the treatment of load-bearing tissues. Nevertheless, hydrogels can be combined with other materials to yield reinforced composite structures with enhanced stability and overall mechanical properties. This approach has been explored with reinforcing structures based on thermoplastic polymers,[51,52] on stiffer hydrogels

printed by fused-deposition modeling,[53,54] or on the incorporation of random microfibers, for example generated by electrospinning.[55] Importantly, these methods showed that approaching the compressive stiffness of native cartilage is feasible.[51,53,54]

Although there are various strategies for the incorporation of multiple materials,[56] the evolution from AM toward biofabrication has shown an advantage over conventional and other AM techniques. This is thanks to the fact that biofabrication uses either multi-head or hybrid printing systems[57,58] that allow both simultaneous and sequential printing, for building up highly organized cell-laden reinforced constructs.[58–63] Moreover, convergence of multiple biofabrication technologies will further extend the possibilities, including the simultaneous deposition of hydrogels and ultra-thin reinforcing fibers produced by melt electrospinning writing (MEW). This technique allows precisely controlled deposition of these micro-fibers, which allows to generate structures with similar compressive behavior as native cartilage.[64]

Additionally, biofabrication provides the opportunity to simultaneously control micro- and macro-architecture of an implant, as precise control can be exerted on spatial arrangement of the framework,[65] which inherently allows control over its mechanical properties. Actually, multiple parameters can be tuned to modulate porosity and compressive stiffness, such as fiber diameter through nozzle diameter or deposition speed, fiber spacing, layer thickness, layer configuration, and fiber orientation.[66–70] In this way mechanical properties can, for example, be influenced by tailoring the local distribution of reinforcing fibers.[68]

Furthermore, for the optimal performance of larger osteochondral implants, it is important that the geometry of an osteochondral implant can be controlled into detail. Consequently, it can provide a perfect fit and alignment with the surrounding tissues to ensure the stability and subsequent integration.[71] Biofabrication can provide such perfectly fitting implants as it allows for the generation of patient-specific anatomical shapes based on digital medical images.[72] In addition, this control over precise geometry provides the ability to design shear resistant surfaces. Taken together, construct shape and mechanical properties can be highly controlled by the application of biofabrication techniques.

Improved Integration

The integration between both the cartilage and bone part of an implant, as well as with the host tissue, is a crucial prerequisite for correct functionality and prevention of graft failure, hence for long-term successful performance of the implant.[39,73] Obviously, this requires a perfect fit and alignment of the construct with the surrounding tissues. Biofabrication technologies could also play a role in improving the integration between the cartilage and the bone, by recapitulating the subchondral bone-to-cartilage transition. This is particularly relevant, as composition, organization and anatomical structure of this interfacial region have important roles in force absorption and transmission.[74] In many conventional tissue engineering approaches, the "interface" is an unintentional by-product of combining the two main parts of the osteochondral construct, which were connected by just press-fitting, suturing, melting, or gluing prior to implantation.[75] However, further insights in the anatomy and function of the osteochondral interface have underscored the importance of proper integration between the bone and cartilage compartments.[76–81] Incorporation of a calcified cartilage zone could improve interfacial shear strength of osteochondral constructs.[82] This interfacial region can also fulfil an important role as a structural barrier to prevent vascular in growth from bone to cartilage.[83]

Biofabrication technologies can pre-eminently yield integrated constructs with various compositions.[84] The simple introduction of gradients in structure (i.e., porosity),[10,85] composition (i.e., minerals and growth factors)[86–88]or mechanics (i.e., stiffness)[27] can influence the differentiation of cells toward bone and cartilage lineages.

Apart from building gradient structures, constructs comprising of a bone and cartilage region can be a simplified mimicry of the native osteochondral unit.[39,89] One method would be depositing layers of the same material to ensure proper axial binding, supplementing it with biofunctional compounds to tune cell behavior in each region. For example, regional distribution of mineral components, such as calcium phosphate nanoparticles[90] and osteogenic micro particles,[91,92] were used to facilitate the osteogenic differentiation in the bone region of bioprinted osteochondral constructs. Furthermore, brittle calcium phosphates can be combined with thermoplastic polymers, like polylactic acid (PLA) or polycaprolactone (PCL) to improve the elasticity of the constructs.[93] Also, composite scaffolds were generated based on layers of electrospun PCL and different concentrations of β-tricalcium phosphate nanoparticles. After 4-week culture, mouse pre-osteoblasts (MC3T3-E1) deposited matrix in a pattern resembling the bone-to-cartilage interface.[94] This approach was also employed to simultaneously fabricate nanofibrous PCL with gradients of insulin and beta-glycerophosphate (β-GP). Human adipose-derived stromal cells differentiated chondrogenically at the insulin-rich sites, while mineralization was predominantly observed in regions where the β-GP concentration was higher.[95] Novel approaches involving the use of advanced biomaterials and the convergence of multiple AM technologies can allow firm integration between different layers even when using heterogeneous components. Recently, strategies to provide strong, covalent binding between hydrogels and polymeric, ceramic, and metallic surfaces have been developed, displaying adhesion forces in the

range of the native bone-to-cartilage interface,[96] which could be adapted for tissue engineering.

Biofabricated osteochondral constructs have been already adopted in an in vivo study involving a MSC-laden collagen and hyaluronic acid hydrogel construct reinforced by PCL. This artificial osteochondral plug was implanted in a rabbit knee and appeared to be mechanically stable and to integrate well with the native cartilage and the underlying bone.[97] The success of this study was, at least in part, due to a perfectly fitting design of the prosthesis, as well as the good integration in both bone and cartilage region, achieved by stack crosslinking with the same cell-friendly chemistry. Although there is still major room for improvement, this example illustrates the potential of biofabrication for optimizing the performance and integration of tissue-engineered osteochondral grafts.

Stereolithographic techniques have been recently combined with extrusion-based AM techniques for application in osteochondral regeneration.[86,98] For instance, an osteochondral unit with a gradual change of mineral composition and growth factors was fabricated using stereolithography. The constructs, composed of a hydrogel with TGF-β1 in the cartilage part and a discrete gradient of hydroxyapatite nanoparticles in the bone part, revealed that differentiation of human MSCs toward the osteogenic and chondrogenic lineages corresponded to the compositional gradients.[86] An appealing approach would be that of combining extrusion of hydrogels, ceramics, and thermoplastics with melt electrospinning writing of nano- and microfibrous meshes. In this way, such meshes could act as interlocking elements between the bioprinted bone and cartilage compartments, to produce a new generation of mechanically stable osteochondral grafts.

Toward Larger Implants and Miniaturized Models: A future Outlook

Biofabrication has the potential to address the challenges mentioned above. It can recapitulate a zonal organization in a graft, it allows for the generation of constructs that approach mechanical properties of native cartilage, and it provides tools for improved integration, both of construct components and with surrounding host tissue. Moreover, it can produce complex shapes in a single fabrication process. Therefore, the technique poses an excellent opportunity to generate larger structures.

A wide range of smaller osteochondral constructs have been successfully generated using AM alone[34,75,99,100] or in combination with conventional techniques, including casting, freeze-drying and solvent casting/particle leaching.[101–104] Even though the generation of a long-term functional solution in osteochondral tissue engineering remains challenging, in vivo approaches with 3D printed osteochondral plugs have already been reported.[97,105–107]

There is now the opportunity to generate larger personal implants, as biofabrication can provide highly accurate anatomical structures[62,108–110] using different materials, either with[111,112] or without[113,114] the aid of a sacrificial support. Feasibility of this concept has been successfully demonstrated in rabbit models, for example, the manufacturing of total knee[72] and humeral head replacements.[115] Nevertheless, there has been limited follow-up on this concept as it is associated with some significant challenges. Some of these are more general and related to the engineering of high quality tissue, while others are specifically related to biofabrication. Cell viability in bioprinting may be compromised, especially during longer printing processes[12] and the process of generating personalized implants in pre-clinical/translational studies is still labor intensive and expensive.[116,117] Also, the establishment of appropriate in vitro pre-conditioning protocols is a time-consuming task[36,118] and the need for post-implantation vascularization should also not be overlooked. While this latter challenge can well be addressed by biofabrication,[119–122] the hollow structures still need to be populated with, for example, endothelial cells.

Biofabrication provides avenues for the generation of larger implants, however, it also offers opportunities for the organ-on-a-chip approach, a technology that aims to simulate specific organ functions and pathologies, and is rapidly advancing in medicine.[123] Models have been developed to mimic a range of different tissue conditions, including alveolar function,[124] intestinal disease,[125] the beating heart,[126] and the blood-brain barrier.[127] In the spirit of the body-on-a-chip initiative,[128] the new concepts of "cartilage-on-a-chip" and "joint-on-a-chip" will be further matured.[129,130] Since the resolution of AM has increased to micro- and nanoscale and progress is made in speeding up the printing process while maintaining its accuracy, biofabrication could definitely also prove itself a key technique in this new area. Ultimately, we envision that patients' chondrocytes or synovial cells could be seeded on a chip, as has been shown in other fields using the organ-on-a-chip technology.[131] The idea would be to determine the immunological profile and gain valuable insights on biomarkers of osteoarthritis, rheumatoid arthritis, and other joint diseases. Similarly, drug efficacy in balancing joint homeostasis could be evaluated.

CONCLUSION

For a successful approach to engineer osteochondral tissue, functional mimicking of this tissue in all its complexity is imperative. To achieve this, one has to address the zonal architecture with a firm connection between different zones and the adjacent host tissue, the biomechanical profile of the native tissue, which is of paramount importance, and a human-scaled personalized shape. For clinical applicability, standardization and possibilities for scaling up are important. Clearly,

promising steps have been taken in vitro to create constructs featuring good integration between bone and cartilage and transition to (large) animal models should now be pursued.

Despite being a relatively new field of technology, biofabrication potentially encompasses all tools and techniques to address these issues and hence is opening promising avenues toward the generation of biologically active personalized osteochondral implants with the ability to regenerate tissue rather than replace it.

AUTHORS' CONTRIBUTIONS

WMG and PD wrote the content of the review. PRvW, RL, and JM contributed to drafting and critically revising the review. All authors have read and approved the final submitted manuscript.

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