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Additive Manufacturing of Biomaterials, Tissues, and Organs

Modular Tissue Assembly Strategies for Biofabrication of Engineered Cartilage

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Abstract—This review describes the prospects of applying modular assembly techniques and strategies for fabrication of advanced tissue engineered cartilage constructs. Articular cartilage is a tissue that has important functions in preserving and enabling locomotion. However, its limited intrinsic repair capacity and lack of current long-term clinical solutions makes it a candidate for repair or regeneration via tissue engineering strategies. Key advances in biofabrication and 3D bioprinting techniques allowing the specific placement of cells and tissues enable novel strategies to be adopted with increased chances of success. In particular, modular assembly, where separate biological components such as microtissue units, cellular building blocks or spheroids are combined with structural scaffold components to create a functional whole, offers potential as a new strategy for engineering of articular cartilage. Various modular assembly or bottom-up fabrication strategies have been investigated or applied for engineering of a range of tissues and cell types, however, modular approaches to cartilage engineering have been limited thus far. The integrative nature of many current approaches to engineering of articular cartilage means optimization of separate components (such as the scaffold and cells) is challenging, resulting in strategies which are less amenable to clinical scale-up or modification. In addition, current tissue engineering strategies may not replicate the function and complex structure of native tissue. This review outlines recent developments in fabrication of cellular or tissue modules as well as scaffold design where it impacts modular biofabrication, and discusses existing modular approaches applicable to articular cartilage regeneration and repair. Modular tissue assembly approaches allow complex hybrid constructs to be fabricated with direct control over both structural and cellular organization of pre-formed tissue units. The combination of modular assembly with automated biofabrication technologies may offer solutions to the development of optimal tissue-engineered cartilage constructs.

Keywords—Biofabrication, 3D bioprinting, Tissue assembly, Cartilage tissue engineering, Cell aggregate, Microtissue, Hydrogel, Microfabrication, Scaffold design.

INTRODUCTION

Articular cartilage enables joint motion and function by protecting the ends of the long bones, allowing movement, providing a lubricated low-friction surface, and absorbing and distributing force. The limited intrinsic repair capacity of articular cartilage is well established, and significant challenges still remain to reliably regenerate damaged cartilage clinically.[13,84] Depending on the severity of injury or disease progression, cartilage damage can manifest as a result of damage to the extracellular matrix, cell death, physical macroscopic disruption of the cartilage surface, damage to the osteochondral interface, and/or damage to the subchondral bone.[13] Under normal circumstances, chondral defects (where damage is restricted to the articular cartilage) fail to heal, and often degenerate over time.[24] While chondrocytes surrounding the defect site do proliferate and can increase matrix production, this is usually insufficient to achieve repair, and the defect remains. Osteochondral defects, where there is infiltration from bone marrow and blood, partially heal with a fibrocartilage repair tissue which has inferior mechanical properties to native hyaline cartilage.[3] This intrinsic fibrocartilage repair tissue further degenerates over time,[43] leading to

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osteoarthritic changes in the joint. Tissue engineering and regenerative medicine approaches using autologous chondrocytes or chondroprogenitor cells hold promise for the repair of articular cartilage defects.[84] This review outlines the potential for use of modular assembly as a strategy for engineering of functional articular cartilage. We outline a modular design paradigm and discuss how developments in 3D model systems, organoid cultures and microfabrication techniques for generating cellular building blocks have produced tissue units appropriate for use in a modular assembly system. We also discuss how the development of automated biofabrication platforms including 3D printing/plotting, 3D bio-printing and complimentary scaffold or microfabrication techniques that produce defined and controllable architectures can be combined into the modular approach and applied to engineered modular cartilage constructs. With these key technological advances, we are now seeing that automated biofabrication of modular constructs is feasible, with potential for scale up.

Modular Assembly

Modularity is a concept that originates from industrial product design. The underlying theory is the use of modules or "building blocks" to combine separate individual components into a functional whole. Modular design is distinct from integral design, where the parts all depend on each other to achieve the desired function.[16] Modular design also imparts the ability to simplify highly complex systems or structures by breaking the overall design into smaller, more manageable parts that can be optimized on an individual basis, and which could not normally be achieved through more integrative approaches. The separate components of a modular product usually have individual functions along with unique features such as size, shape, or interfaces that allow them to be coupled or assembled together.[47] Ideally, different modules should be able to be exchanged, allowing separate development and optimization, and the fabrication of modules themselves may also allow for scalability.

Modular tissue assembly can be defined as a method for step-wise biofabrication of larger tissue constructs in 3D using defined tissue units or cellular building blocks. The modular assembly approaches referred to here could be considered a combination of bio-printing, bio-assembly and classical tissue engineering (as defined in Groll *et al.*[33]). It combines traditional tissue engineering approaches using a cell-seeded scaffold with the use of defined or pre-formed tissue units. Often modularity is not directly considered in tissue engineering and regenerative medicine, and integrative design approaches are more common.[63] This is most likely due to the complex, interconnected systems involved in the development of functional engineered tissue consisting of living cells, which in fact could be considered as a system of components and functions that cannot be separated. On the other hand, this very complexity indeed supports the case for modular approaches, as beginning with simple reducible modules, and combining these to assemble systems of increasing complexity may offer significant advantages.

Examples of Modular Tissue Assembly

Three-dimensional assembly of tissue units has been attempted with a number of different cell and tissue sources, with the target application ranging from simple to highly complex tissues such as trachea, blood vessels, dermis, cardiac tissue, osteochondral tissue and hepatic tissue (see Fig. 1; Table 1). As outlined in Table 1, applications and target tissues for modular assembly have ranged from microfluidic-based 'tissue-on-a-chip' type organization of tissue units[12,40] and investigation of the shaped aggregation of different cell types,[21] to blood vessel fabrication,[41,51] cardiac tissue models[22] and repair patches,[137] dermal tissue approaches,[92] hepatic tissues,[93] airway systems,[129] trachea,[23] and fabrication of bone constructs.[71] Liu and Gartner[68] as well as Gauvin *et al.*[31] have previously reviewed the range of methods available and examples for microscale tissue module fabrication.

Aggregate or spheroid-based tissue modules are particularly attractive, both as a route to modular assembly of tissue organoids, and as 3D *in vitro* models for high-throughput screening. These approaches have witnessed huge growth recently in the application of high-throughput 3D models for drug screening.[74] This is largely due to the ability to use aggregates and/or spheroids containing multiple cell types (i.e. co-cultures).[96,101] It is possible to control the cell aggregation process to allow the formation or self-assembly of microtissues with defined or complex shapes.[34,48,49,65,69,76,77,89] Approaches to achieving this controlled formation of shaped tissues includes methods for forming aggregates or microtissues via pellet culture via hanging drop,[117] centrifugation,[73,107] simple aggregation,[128] or the use of micromolds,[88] non-adhesive culture surfaces[135] or bioreactor systems.[30,104] In addition, molds or micromolds, random packing of modules, and directed assembly can be used to control overall assembled tissue shape using aggregates alone or in combination with supporting structures to direct shape and organization, as outlined in previous reviews.[49,89] Mold casting with aggregates has also been demonstrated with chondrocyte- and C2C12-based aggregates,[48] and Livoti and Morgan[69] used

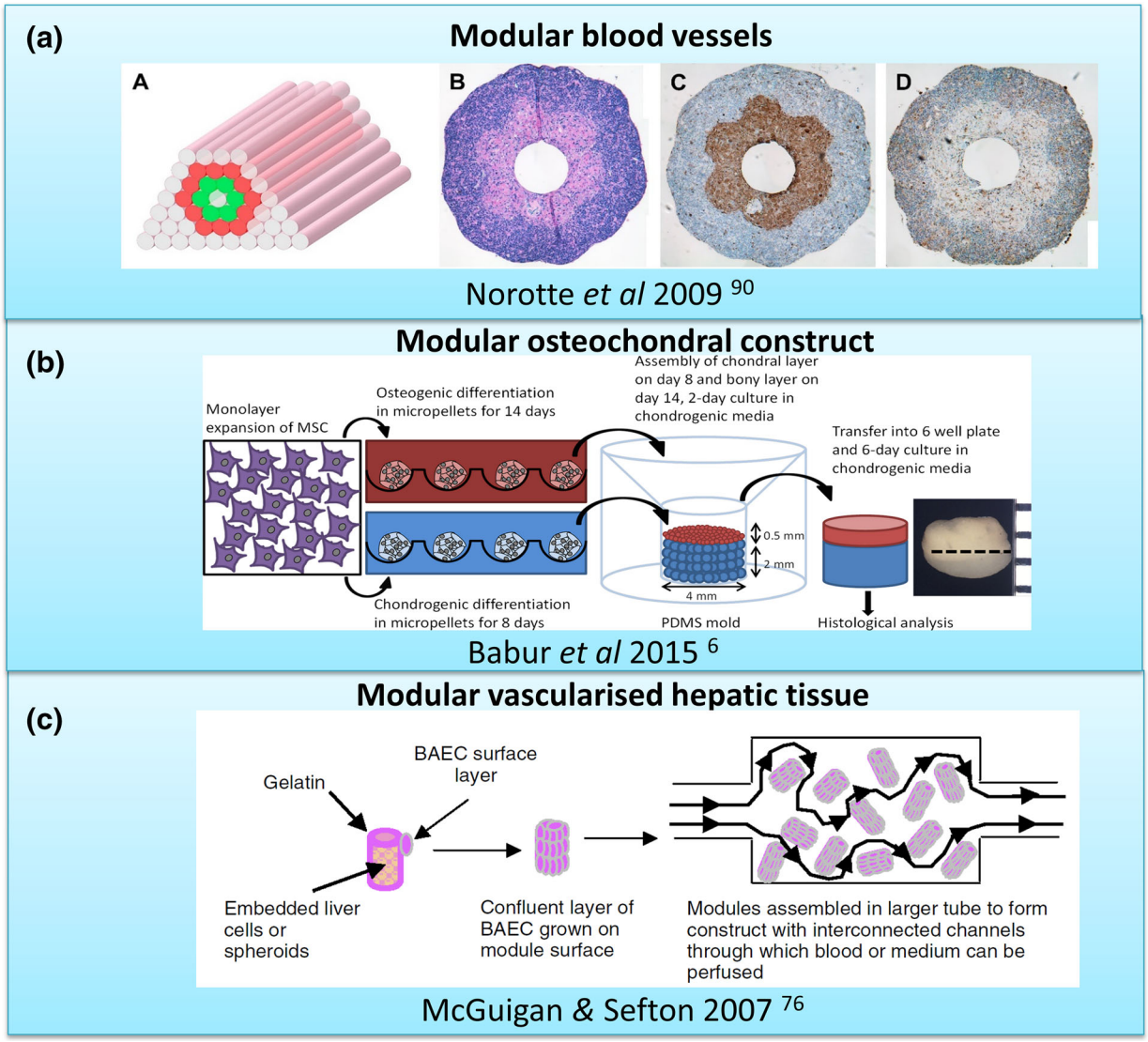

FIGURE 1. Examples of modular assembly applications: Panel A—blood vessel assembly using aggregates with supportive hydrogel,[90] Panel B—osteochondral assembly using pre-differentiated MSC aggregates,[6] Panel C—assembly concept for vascularized hepatic organ using gelatin-based modules.[76]

agarose molds to demonstrate the formation of toroid-shaped tissue units. By stacking and subsequent fusion of the toroids with prolonged culture, they demonstrated it was possible to assemble tubular shapes aimed at generating a vascular construct. Dikina *et al.* have used a similar approach to engineer a trachea-like tissue.[23] A directed 'printing' approach has also been applied to spheroid aggregation. After observing that *ex vivo* chick embryo atrio-ventricular tissue fragments fused together and formed spheroids, Jakab *et al.*[44] fabricated a number of 500 μm diameter cylinders composed of cell aggregates, which were subsequently cut and allowed to round into spheres These microtissues were suspended within a hydrogel and assembled in 3D using an automated printing process, with the goal of promoting tissue fusion and the generation of organoid-like structures using a bioprinting or biofabrication approach. The potential of this approach using spheroids as building blocks for tissue fusion and organ printing has also been discussed by Mironov *et al.*[82] Encapsulation of aggregates or microtissue modules within hydrogels has also been adopted, for example by casting of micro-aggregates of cartilage within a hydrogel.[123] Alternatively, tissue units consisting of cell-laden microcarriers have been encapsulated or combined with 3D bioprinting of hydrogel fibers or scaffolds for tissue engineering applications.[66]

Modules may not necessarily have to be prefabricated into a construct, and can also be assembled *in situ* at the site of the tissue defect. For example, scaffold free delivery of microtissues or chondrospheres alone have been examined for cartilage

TABLE 1. Examples of modular assembly approaches demonstrating a variety of target tissues.

Author	Cell type(s)	Application or model
Bruzewicz *et al.*[12]	3T3, HepG2	Demonstrated assembly of cell-containing gel modules on microfluidic " tissue on a chip"
Caldwell *et al.*[15]	hMSCs	Centrifugation and vacuum moulding used to make multiphase layered constructs using collagen I-chitosan beads, some including hydroxyapatite
Dean *et al.*[21]	NHFs, H35 s	Cells seeded onto micromoulded agarose gels, in order to produce shaped aggregates for future assembly
Desroches *et al.*[22]	Cardiac myocytes, cardiac fibroblasts	Investigated cardiac 3D model using micromoulded agarose to form microtissues
Dikina *et al.*[23]	Human MSCs	Toroidal aggregates stacked and stimulated to form trachea-like structure
Gupta *et al.*[34]	HUVECs	Transplanted assembled constructs of HUVEC coated cylinders into rats
Imparato *et al.*[40]	Human dermal fibroblasts	Crosslinked porous gelatin microbeads used to make dermal tissue for model "tissue on chip".
Inamori *et al.*[41]	Rat hepatocyte, HUVECs	Rat hepatocyte spheres fabricated, coated with HUVECs, assembled, perfusion cultured
Kelm *et al.*[51]	Human artery-derived fibroblasts, HUVEC	Tissue-engineered blood vessels based on HAF and HUVEC microtissues
Leung and Sefton[65]	rAEC, rat cardiomyocytes	Cardiac modules formed, for engineering vascularised cardiac tissue
Maiqin *et al.* [71]	Amniotic MSCs	Microcarriers + amniotic MSCs used to make large construct, for formation of bone tissue
Norotte *et al.*[90]	CHO, HUVSMCs, HSFs	Fabricated blood vessel constructs using multiple cell types via bioprinting
Palmiero *et al.*[92]	Dermal fibroblasts	Aimed to assemble dermal equivalents by assembling aggregates formed on gelatine microcarriers
Pang *et al.*[93]	Rat hepatocytes, HUVECs	Used PDMS mould to form aggregates, combined with PLLA fibres and perfused for liver tissue
Rago *et al.*[101]	Hep2G	Demonstrated method for encapsulating microtissue in alginate as alternative to microencapsualtion of monodispersed cells
West *et al.*[129]	Airway smooth muscle cells, 3T3 fibroblasts	Airway smooth muscle model system. Array of microtissues formed and studied
Zimmerman *et al.*[137]	Primary neonatal rat heart	Engineered cardiac tissue formed, exercised, modules combined to repair infarcted hearts *in vivo* in rats

3T3 mouse fibroblast cell line, *Hep2G* human hepatocarcinoma cell line, *CHO* Chinese hamster ovary cells, *HUVSMCs* human umbilical vein smooth muscle cells, *HSFs* human skin fibroblasts, *hMSCs* human mesenchymal stromal (stem) cells, *NHF* normal human fibroblasts, *H35* rat hepatoma cell line, *HUVEC* human umbilical vein endothelial cells, *HAF* human arterial-derived fibroblasts, *rAEC* rat aortic endothelial cells.

repair,[61,80,109] and injectable micro-aggregates combined with an injectable hydrogel have been suggested for use in improving on existing cartilage repair procedures such as MACI (matrix-induced autologous chondrocyte implantation).[123] When compared to traditional tissue engineering strategies involving delivery of single cell suspensions, microtissue delivery may have an advantage in that they are more easily localized at the defect site due to their larger size and extracellular matrix (ECM) production, while still maintaining an injectable formfactor.[50]

In addition, cell sheet technology has been used in modular approaches, either providing a number of different cell layers or in combination with a scaffold, and can be included in a layer-by-layer biofabrication strategy.[19,98,105] Modular assembly has also been demonstrated using combinations of pre-differentiated tissue units. For example, Zimmerman *et al.*[137] demonstrated the fabrication and *in vivo* application of an assembled cardiac patch consisting of several relatively large (~10 mm length) rubber band-like pre-differentiated and exercised modular units, which were able to partially rescue cardiac function in a rat myocardial infarction model. The assembly of chondrogenic microtissue units within the pores of structural 3D plotted polymer scaffolds has also been demonstrated.[107,111] The production and assembly of cell-laden hydrogels with monodisperse cells encapsulated in the hydrogel is also a popular approach. Examples of hybrid hydrogel-based approaches aimed at modular assembly include: vascularized assembled tissues around gelatin-based modules for various target cell types and tissues,[14,34,52,64,65,76,78] multiphasic constructs formed from collagen-chitosan beads (with some including hydroxyapatite),[15] and controlled assembly of various gel-based modules to generate specific 2D or 3D structures.[25,26] Design criteria for a random packing approach (as shown in Fig. 1, Panel C) has been outlined,[77] and random packing of modules has been demonstrated in modular cardiac tissue.[65] Random packing has also been used to generate

cardiac-like constructs assessed *in vitro*,[65] as well as endothelialized or vascularized constructs which have been assessed *in vitro*[64,76] and *in vivo*.[34]

CURRENT APPROACHES FOR TISSUE ENGINEERING OF ARTICULAR CARTILAGE

The traditional tissue engineering paradigm typically involves the *ex vivo* expansion (and concomitant dedifferentiation) of chondrocytes or chondroprogenitor cells that are subsequently placed into a 3D environment, in conjunction with appropriate growth factors and cytokines to induce differentiation and extracellular matrix (ECM) production.[130]

A number of tissue engineering approaches to date have focused on the regeneration of articular cartilage and often have adopted the use of either a 3D scaffold or cells, or a combination of the two, to elicit repair. Recommended design criteria for tissue engineered construct development have been established, and include: (1) efficient seeding and homogeneous distribution of cells, (2) high porosity and 100% interconnected pore network, (3) promotion of a differentiated cell phenotype and ECM formation, (4) high cell viability, (5) appropriate mechanical properties to achieve tissue function, and (6) integration with the surrounding host tissue.[56,113] These are also considered to be important criteria for successful tissue engineering of articular cartilage.[130]

One of the reasons why articular cartilage is so difficult to repair or regenerate is due to its deceptively complex zonal organization, with the tissue exhibiting variations in ECM composition (e.g. proteoglycan content and collagen type), collagen fibril orientation, and depth-dependent cell organization and phenotype[3] (as illustrated in Fig. 2, panel A). A number of methods have attempted to mimic or recapitulate the zonal architecture of cartilage (as reviewed by Klein *et al.*[55] and Tatman *et al.*[121]). Strategies include: seeding cells of different zonal origin into appropriate regions within the construct, using decellularized native ECM or gradient scaffolds that mimic aspects of the zonal organization,[133] or provision of gradients in chemical or physical cues to induce appropriate differentiation.[55] Furthermore, scaffolds may be fabricated with gradients in composition and/or architecture in order to mimic mechanical properties of native cartilage and allow the distribution of appropriate mechanical cues to cells throughout the different zones. In addition, combinations of fabrication techniques have been used to provide multiscale cues and appropriate mechanical

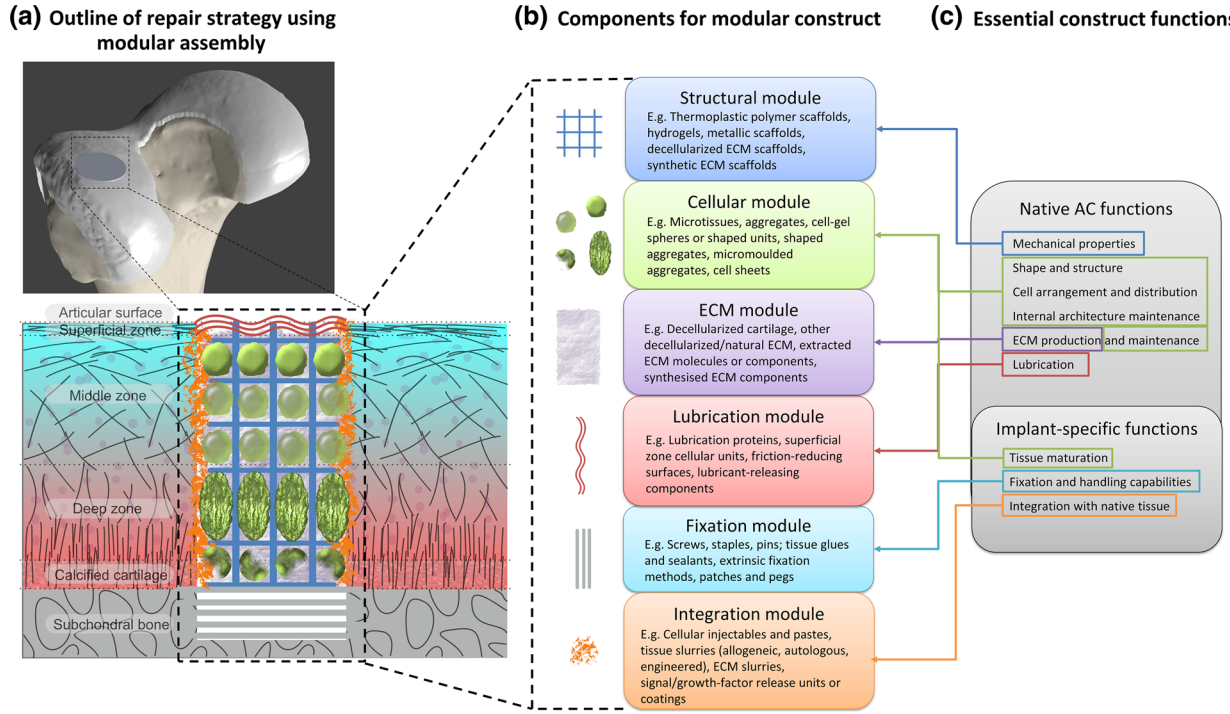

FIGURE 2. Schematic illustrating potential strategies for cartilage regeneration adopting modular assembly approaches. Essential functions of the engineered construct are achieved by the development and delivery of individual modules. Panel C outlines the functional properties inherent to native articular cartilage (AC). Panel B describes the separation of essential tissue functions into respective modules for individual optimization and assembly. The lower portion of Panel A shows the modular construct assembled in the native tissue, demonstrating how these individual modules can be combined and applied to the repair of chondral or osteochondral defects as shown in the upper portion of Panel A.

properties throughout engineered cartilage constructs.[121]

ARTICULAR CARTILAGE ENGINEERING STRATEGIES

Scaffold-Free Cell Aggregates and Bio-Assembly Approaches

Concern about material reactions, interactions and interference with repair procedures have led to investigations of completely scaffold-free, fully biological methods for engineering cartilage tissue. Scaffolds, while having the advantages discussed subsequently, are unable to respond to the environment in the way living tissue can, and many have in the past caused foreign body reactions with rejection of the implant or formation of a fibrous capsule.[50]

Scaffold-free approaches to cartilage tissue engineering have been investigated, and several studies have demonstrated neo-cartilage formation in large aggregates, often using a membrane or transwell-based culture method.[60,75,86,94,99] These results have often generated hyaline-like tissue *in vitro*, however, some of these studies[94,99] have used primary, non-expanded chondrocytes, which would be difficult to obtain in sufficient numbers in humans and consequently unlikely to be of value in clinical practice. Promising scaffold-free results have been obtained with MSCs in transwells,[86,87] and in combination with calcium polyphosphate substrates to form biphasic constructs,[60] however, the mechanical properties were still inferior to native tissue, and durability and success *in vivo* is yet to be determined. Scaffold-free tissue spheroids have been applied directly as an injectable therapy, with several *in vivo* studies demonstrating short-term repair success.[61,80,109] However, in the case of many scaffold-free approaches, concerns exist with respect to whether sufficient mechanical properties can be achieved in scaffold-free constructs and how this will affect viability and long-term function *in vivo*.

Scaffold-Based Repair and Biofabrication Approaches

Scaffolds for engineering of articular cartilage may refer to synthetic- or naturally-derived biomaterials used to support fabrication of a tissue-engineered construct or assisting in inducing repair from circulating host cells (e.g. via localized delivery of growth factors or cytokines including cell homing). Factors to consider in scaffold fabrication include material composition, macrostructure and microstructure of the scaffold, pore size, porosity and interconnectivity, scaffold surface area, mechanical properties and degradation characteristics.[80] Bulk porosity and pore interconnectivity are important, as they assist with homogenous cell adhesion and seeding, and allow nutrient transfer as well as extracellular matrix production.[100] Fabrication methods and their advantages and disadvantages are outlined in a number of reviews.[18,28,38,79,100]

Scaffold-only repair of articular cartilage is an attractive approach and has the distinct advantage of requiring no donor tissue or cells. This strategy eliminates problems of donor tissue morbidity or restricted cell supply while usually providing a single-surgery solution, as well as avoiding cost and difficulty of *ex vivo* expansion. Scaffold-only approaches rely on endogenous repair, and the scaffolds used may have factors or design features included to enhance chondrogenesis. However, it is challenging with scaffold-only approaches to control the composition and cell types infiltrating the scaffold, or the arrangement of an organized extracellular matrix. Despite some promising results in both animal models[27,29] and humans,[114] systematic long-term evaluation of these strategies is required to demonstrate improvements over existing repair strategies.[28]

When a strategy combines cells with a scaffold, as in biofabrication or traditional tissue engineering strategies, the scaffold must be designed to promote chondrogenic differentiation and extracellular matrix development, either *in vitro* or *in vivo*. The scaffold performs further roles in structural support, as well as promoting cell adhesion and/or attachment. Scaffolds are often designed to include differentiation or homing factors or other biological cues which when combined with current advanced 3D printing or biofabrication strategies have allowed large, anatomically shaped tissue-engineered cartilage constructs to be developed for evaluation *in vivo*.[54,131] Commonly reported challenges with cell-scaffold based approaches include difficulties with cell seeding density, uniform cell distribution, and lack of control of cell distribution and location.[130] Cell seeding efficiency may be low, depending on seeding regime or donor variability. Non-homogenous seeding may also be an issue, particularly in larger and less porous scaffolds. Cell attachment can be enhanced by surface functionalization or appropriate selection of scaffold biomaterial,[132] however achieving large, clinically relevant sized constructs while relying on cell attachment and spreading results in de-differentiation and limits ECM formation.[9,20,73] While hydrogel-based scaffolds or constructs allow for diffusion of nutrients and soluble factors, and help retain and distribute cells, embedding cells within a hydrogel results in reduced cell–cell interactions, which can affect gap junction signaling,

re-differentiation capacity and matrix production.[9,108] Hydrogel-based constructs that promote chondrogenesis also tend to exhibit inferior mechanical properties compared to native articular cartilage,[116] and represents a significant challenge for their successful application *in vivo*. For example, there are risks associated with hydrogel integrity and fragmentation under loading, as well as impacting cell viability and quality of repair tissue. Decellularized cartilage ECM has also been investigated by a number of authors as a potential scaffold (as outlined in Sutherland *et al.*,[119] Benders *et al.*[8]) including use as a bio-ink.[95] However, decellularization of articular cartilage ECM without significant loss of glycosaminoglycans (GAG) is particularly challenging, as is the maintenance of collagen type II integrity and organization.[53] In general, decellularized ECM has the advantage of having an abundant supply of raw material, as well as providing cues and signaling molecules inherent in the native tissue. For example, a decellularized ECM-based product is currently available for use in humans (Chondrofix® Osteochondral Allograft), though long-term success of this repair method is still to be determined.[119]

More recently, there has been significant attention focusing on the development of hybrid constructs through the combination of biofabrication technologies that synergistically provide an ECM-like environment for cells, with structural scaffold components for mechanical stability and function *in vivo*. For example, several studies have combined melt dispensing of structural degradable polymers (e.g. poly-ε-caprolactone, PCL) via 3D Plotting with bio-printing of a hydrogel or bio-ink component, with or without cells.[42,59,91,127] A popular fabrication approach has used 3D bio-printing to co-localize the hydrogel and structural scaffold components in a layer-by-layer manner, or in particular hybrid systems which combine hydrogel dispensing/casting with melt electrospinning-writing,[126] or inkjet printing with electrospinning.[126,134] Hydrogel mechanical properties may also be improved by inclusion of particles in combination with cells within the hydrogel matrix, such as PLA microcarriers.[66] In addition, ECM or decellularized ECM hydrogels have been developed and biofabricated in combination with structural scaffold components (e.g. PCL) to deliver properties relevant to cartilage and subchondral bone regions for osteochondral tissue engineering.[95,112] These hybrid approaches have enabled the development of constructs with improved mechanical properties and ECM organization in combination with cell-friendly substrates.

A range of key design criteria therefore need to be controlled or identified when considering scaffold-based or scaffold-free approaches for tissue engineering of cartilage. There are also a number of issues that are yet to be solved in terms of developing successful cartilage repair strategies clinically. Recent advances in technology platforms such as additive manufacturing and biofabrication have been developed to manipulate and assemble scaffolds and/or cells in 3D, and the role of these strategies in modular assembly are discussed as follows.

MODULAR ASSEMBLY OF CARTILAGE TISSUE

A modular tissue assembly approach for cartilage repair allows the combination of components of cartilage into a larger or multi-functional construct for implantation. An assembled tissue would, ideally, have all the attributes of the optimal tissue engineered structure, including appropriate function (e.g. mechanical properties, geometry, tissue quality), be able to integrate with the surrounding native tissue, avoid immunogenicity, and have good biocompatibility. The key role of the construct would be to generate a tissue where function is indistinguishable from native cartilage, and that is able to maintain this function over the long term. An optimal approach may also involve the maximization of cell–cell interactions and seeding efficiency in 3D, stimulating cartilage extracellular matrix production and a re-differentiated phenotype, and the minimization of detrimental cell-material interactions that result in cell spreading and de-differentiation.

Modular assembly methods can improve seeding efficiency, allow delivery of growth factors and cytokines to cells that can be spatially arranged using automated biofabrication processes, and provide an avenue for pre-formed ECM to be introduced into the scaffold. In this way, replication of the complex zonal organization of native articular cartilage could also be addressed with the aim of achieving functional repair.

A concept for the future development and fabrication of engineered constructs for cartilage repair adopting modular assembly is outlined in Fig. 2. Here, the well-established functional criteria required for successful development of an engineered construct and cartilage repair strategy are defined and separated into independent modules. The separate structural, cellular, ECM, lubrication, fixation, and tissue integration modules are outlined in Panel B, and collectively provide the tissue construct functions described in Panel C. In this strategy, modules are able to interact and interface with one another and, when combined with automated biofabrication technologies, they collectively provide the essential construct functions (Panel C) in an *in vivo* cartilage repair process as shown in Panel A. An alternative or simplified approach is

outlined in Fig. 3, where the key functional criteria of articular cartilage are provided by several, as opposed to many, modules. Similar approaches can be used for fabrication and modular assembly of tissues other than articular cartilage.

Development of Tissue Modules for Biofabrication of Articular Cartilage

Successful fabrication of tissue modules that promote differentiation of chondrocytes or chondroprogenitor cells and generate cartilage-like ECM is one of the steps toward modular assembly. Spheroidal microtissues are well-established, useful and straightforward method of producing cartilaginous tissue. Cartilage microtissues may be easily aggregated by micro-mass pellet culture, including hanging drop, centrifugation, or simple condensation of high-density cell suspensions,[73,115,128] and these have been shown to produce hyaline-like cartilage tissue in small quantities.[45,136] Culture in chondrogenic media then allows the chondrocytes to condense and form a spheroid within 48 h. By promoting high numbers of cell–cell interactions and the replication of the 3D cell condensation environment typical during developmental stages of cartilage growth,[1,7,72,120] chondrocytes cultured in pellets produce hyaline-like neo-cartilage *ex vivo* expressing key chondrogenic markers.[45,136] Microtissue spheroid formation in various cell types have been investigated; including primary articular[73] and nasal chondrocytes, bone marrow- and adipose-derived multipotent progenitor cells,[39,46,83,85,125] as well as immortalized cell lines.[120] Different species have also been used as cell sources; including pigs,[32,70] cows,[11,17] goats,[81] rabbits,[36,124] horses,[110,125] chickens[136] and humans.[35,45,72,73,83,85,103,115,122]

While there has been some investigation into assembling larger engineered tissues using aggregated spheroids[48,60,80] or clinical approaches directly filling cartilage defects with pre-formed spheroids,[61,80] the evidence is not convincing that spheroids alone are capable of forming fully functional articular cartilage *in vivo*. Spheroids will likely require support (or surgical 'decompression' in the form of an unloading osteotomy) during the initial implantation stages and tissue maturation to shield the immature tissue from direct mechanical compression under joint loading, since mechanical loading of cartilage in known to induce cell death and disrupt the ECM.[118] The lack of mechanical support in some existing articular repair strategies may be part of the reason for the inconsistent and relatively poor results of current therapies.[56]

Modular tissue units of a variety of shapes and length scales (e.g. from 1 to 2 cells up to 1–2 million or more cells) may also be used, though clearly they would need to be designed to fit with the corresponding scaffold or structural modules they will be assembled with. An example of an automated biofabrication strategy adopting modular assembly approaches—primarily applying the use of spherical tissue units and 3D Plotted

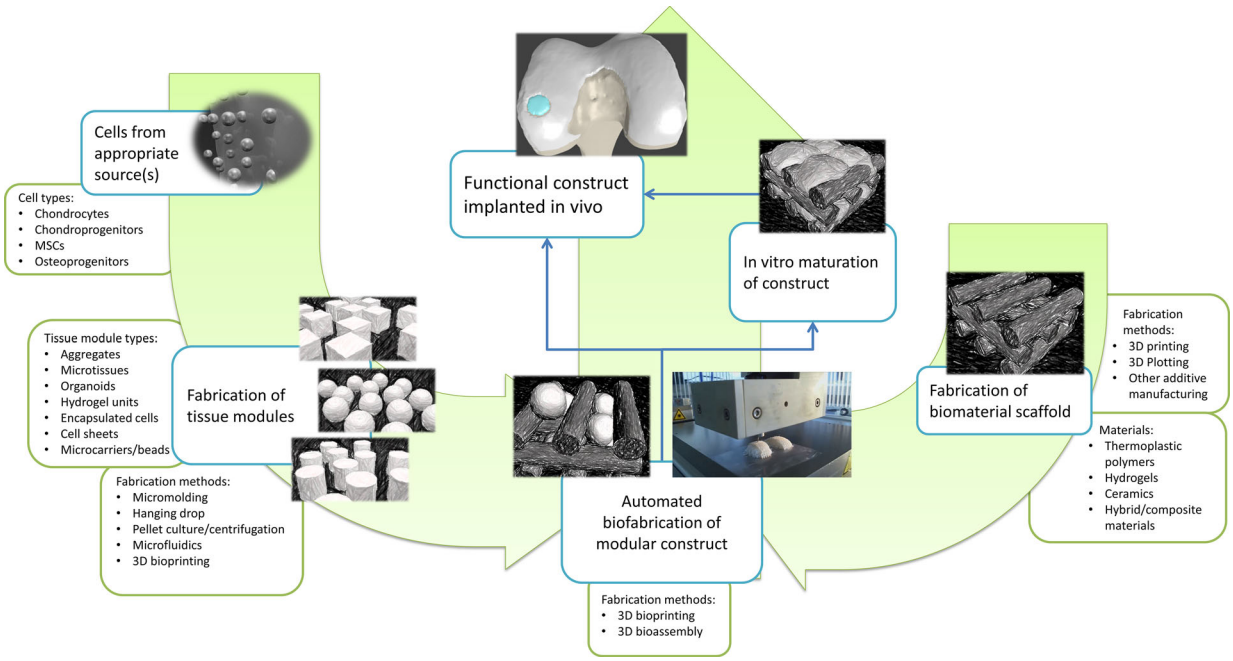

FIGURE 3. Outline of a biofabrication platform for modular assembly of tissue engineered cartilage using tissue and scaffold modules.

thermoplastic polymer—is outlined in Fig. 3. Shaped aggregates amenable to modular assembly may be fabricated by photomask or hydrogel -based methods, or using micro molds, as reviewed by Zorlutuna.[138] Alternatively cell-sheet modules can be mass fabricated for inclusion in a layer-by-layer biofabrication strategy.[19,98,105] Tissue formation in membrane cultures has been previously demonstrated,[60,75,86] in addition to formation of large scaffold-free aggregates.[94,99] Whether these could be combined with scaffold or structural modules remains to be seen, and may prove difficult.

Structural Scaffold Fabrication

There are three critical parameters for scaffold design when using a modular approach: scaffold function, interface with other modules, and independence. The function of the scaffold must be fulfilled by appropriate design and fabrication. In the case of modular articular cartilage constructs, this function may be solely structural, or may incorporate a number of the functions outlined in Fig. 1, such as structure and mechanical properties, lubrication, ECM, fixation; or can be designed to accommodate these separate modules (as shown in Fig. 3). The internal and external architecture will vary depending on the type and size of other modules used and the specific application and anatomical location. The scaffold must allow for interface with the other modules, such as tissue units (for example as in Fig. 3) and must be scalable to generate a clinically reagent-sized construct. It must also be somewhat independent from these modules to allow for independent optimization.

Additive manufacturing techniques such as 3D printing and 3D plotting are now well-established and advanced scaffold fabrication technologies offering advantages for the controlled manufacture of complex 3D structures with defined pore architectures and mechanical properties.[49,54] Size, shape, porosity and interconnection can be controlled, and scaffolds can be scalable and fabricated from biocompatible, tunable materials. The scaffolds are highly reproducible, and mechanical properties can be altered through controlled modification of scaffold architecture. Interfaces with tissue units can therefore be designed into the scaffold, and these techniques also allow for substitution of materials by with adjustment of fabrication parameters. For example, 3D plotting makes it possible to design scaffolds with pore architecture or fiber spacing that allow insertion and bio-assembly of spheroidal microtissues of the appropriate dimensions[57,58,106,107,111] (see Fig. 3). Babur *et al.* have demonstrated both formation of cartilage micropellets using cartilage 'dust' as a substrate,[6] as well as assembly of chondrogenic and osteogenic micropellets into a biphasic construct, demonstrating fusion and tissue formation of the layers *in vitro*.[5] In addition, an approach using microcarriers to generate microtissues that could be extruded within a hydrogel 'bio-ink' has been demonstrated by Levato *et al.*,[66] including bioprinting of biphasic constructs consisting of chondrogenic and osteogenic regions, and a bioprinted biphasic scaffold has also been demonstrated along with implantation in a rabbit model.[112] Adipose-derived mesenchymal stem cell spheroids assembled in a PLGA scaffold have also been applied in a chondral defect model in rabbits.[37] This study showed that by varying the substrate composition and culture conditions used for spheroid formation had an impact on the quality of *in vivo* cartilage repair at 1 month.

ASSEMBLY OF ARTICULAR CARTILAGE: CONSIDERATIONS AND PROMISE

Assembling pre-differentiated cartilage micro-tissues within scaffolds offers the ability to precisely control the locations of tissue units, enabling the fabrication of complex, organized 3D tissues. Given that the basis for many tissue assembly approaches involves automated or high-throughput fabrication methods, such a strategy would potentially allow for the automated manufacture or biofabrication of large cartilage implants. It also allows for a high scaffold porosity to be achieved due to the high degree of control inherent in the fabrication process, while maintaining a high cell density and high seeding efficiency,[106,110] which is not easily achievable using more traditional tissue engineering approaches adopting seeding and differentiation of single cell suspensions within a porous 3D construct. Theoretically, the upper size limit of modular assembly will be based only on the total number of cells obtained via biopsy and the chosen cell density used during tissue module formation. Cell expansion and associated limitations in generating very large numbers of cells must be considered, however, and studies have shown that co-culture of primary cells (e.g. MSCs harvested in surgery with primary chondrocytes) could be an alternative to relying on mass expansion for forming sufficient numbers of tissue units.[2] A range of MSCs have been investigated for potential cartilage repair,[62] however depending on the source, there may be limitations on chondrogenic potential. There are concerns that the cells tend to undergo hypertrophy[62] and may progress to mineralization[85] though this may be reduced by using co-culture with chondrocytes.[2,10] Articular cartilage is a complex tissue consisting of distinct subpopulations of superficial, middle and deep zone chondrocytes, with each zone exhibiting specific cell orientations as well as

an organized extracellular matrix with unique zonal properties and composition.[4,97,111] Tissue assembly approaches could be applied either to recreate these zonal subpopulations, or could be used to form robust tissue that can be implanted to create a more optimal extracellular matrix microenvironment for the chondrocytes to function as in native tissue. It should be noted that an initial attempt to recapitulate zonal organization in assembled cartilage constructs using pellet-cultured microtissues did not achieve zonal organization *in vitro*.[111]

Interdependence of components can make modification and optimization of tissue engineered constructs difficult,[63] suggesting that a truly modular design must ensure that the components used are not completely interdependent. The use of a modular bio-assembly method for cartilage formation may reduce interdependence and enable individual optimization of the tissue quality and scaffold properties, and allow enhanced constructs to be fabricated. Furthermore, these components could be separately developed, enhancing the overall construct without complete redevelopment. For example, a tissue module may be developed initially around pellet culture, but may move to a bioreactor or micromolding approach to provide automation and scalability. Meanwhile, the scaffold material could be tuned to enhance mechanical properties. These developments could them be re-incorporated into the modular construct to enhance the overall construct function. Developments in the choice of cell sources, module fabrication methods, or new alternative scaffold materials may therefore be more easily incorporated within a modular (as opposed to an integral) engineered construct.

Alternative Cartilage-Related Clinical Applications

There are a number of applications other than articular cartilage whereby modular tissue engineering approaches has been adopted, and the use of cellular modules based on different cell types could allow for a range of different tissue constructs to be formed.

Structural cartilage in non-articular locations within the body may be fabricated using the modular assembly method. For example, specific applications include septal cartilage replacement, and patient-specific shaped cartilage, bone, or osteochondral craniofacial and maxillofacial implants for facial reconstruction.[67] Trachea is another cartilage-based structural application where assembly may find favor, and automated assembly and structural module fabrication will be advantageous in these application. By altering the scaffold properties, mechanical properties of the construct can be modified, and a more "elastic-like tissue" could be fabricated, making modular assembly suitable for elastic cartilage structures, such as auricular applications.

Applications of Modular Tissue Assembly: In Vitro Models and Screening

There is still much to be understood about aspects of articular cartilage biology and underlying cellular mechanisms of chondrocytes and chondroprogenitor cell differentiation inherent to developing successful tissue engineering cartilage repair strategies. While simple two-dimensional (single-cell or monolayer) *in vitro* models and complex *in vivo* models exist, there is a need for the development of 3D models of intermediate complexity[102] that better predict *in vivo* environment or allow for high throughput analysis of the plethora of cell–cell or cell-biomaterial interactions that may control tissue formation and cell differentiation processes. Besides its clinical potential, modular assembly provides a 3D model that allows for investigating a number of important topics, with the modules providing an extension of a well-established chondrogenic culture technique. *In vitro*, interventions can be used to investigate the roles of various factors on cell and tissue interactions, such as soluble factors, mechanical stimulation, cell-biomaterial and biomaterial-ECM interactions. High-throughput screening could be a useful application for the modular assembly method described here. Tissue modules of different types could potentially be organized into 3D *in vitro* or *in vivo* mini-organ cultures or organoids comprised of organized cartilage or osteochondral constructs, which would represent an intermediate step between 2D cell cultures and whole organisms. Organoids have been used for investigating tissue mechanisms, in cancer biology and for high-throughput screening purposes, and this could inform future use of these models in research for cartilage. Automated assembly and handling of these constructs *in vitro* could be an attractive and cost-effective method for a relevant 3D model system for screening drugs or compounds for treatment of disease, as well as factors for enhancement of regeneration. *In vivo*, the scaffold-plus-spheroid model could be used to apply a matrix-style or mini-organ style construct setup for testing multiple cell types or conditions within a single implant.

CONCLUSIONS

Modular assembly is a relatively unexplored approach to articular engineering that presents exciting advantages. Modular approaches to assembling a range of different tissues have been investigated, however only limited demonstration of articular

applications have been shown. The ability to separately optimize and develop tissue and structural modules may be advantageous, with development and improvement of the components of the construct not relying directly on each other. Further technological and other developments could therefore be integrated into the construct more quickly.

The use of existing models and methods for fabrication of modules presents an opportunity of applying models to a larger construct environment, and a variety of tissue and structural modules could be combined if the interface and physical properties of the components are carefully considered. Ensuring the modules fulfil both a modular function and appropriate role within the cartilage will provide advantages for future development and optimization of the construct. The future of cartilage regeneration and repair may indeed lie in development of individually optimized, interacting, interchangeable modules that deliver all the requirements for a fully functional articular construct.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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