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Review article

Biofabrication and bioprinting using cellular aggregates, microtissues and organoids for the engineering of musculoskeletal tissues

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ABSTRACT

The modest clinical impact of musculoskeletal tissue engineering (TE) can be attributed, at least in part, to a failure to recapitulate the structure, composition and functional properties of the target tissue. This has motivated increased interest in developmentally inspired TE strategies, which seek to recapitulate key events that occur during embryonic and post-natal development, as a means of generating truly biomimetic grafts to replace or regenerate damaged tissues and organs. Such TE strategies can be substantially enabled by emerging biofabrication and bioprinting strategies, and in particular the use of cellular aggregates, microtissues and organoids as 'building blocks' for the development of larger tissues and/or organ precursors. Here, the application of such biological building blocks for the engineering of musculoskeletal tissues, from vascularised bone to zonally organised articular cartilage, will be reviewed. The importance of first scaling-down to later scale-up will be discussed, as this is viewed as a key component of engineering functional grafts using cellular aggregates or microtissues. In the context of engineering anatomically accurate tissues of scale suitable for tissue engineering and regenerative medicine applications, novel bioprinting modalities and their application in controlling the process by which cellular aggregates or microtissues fuse and self-organise will be reviewed. Throughout the paper, we will highlight some of the key challenges facing this emerging field.

Statement of significance

The field of bioprinting has grown substantially in recent years, but despite the hype and excitement it has generated, there are relatively few examples of bioprinting strategies producing implants with superior regenerative potential to that achievable with more traditional tissue engineering approaches. This paper provides an up-to-date review of emerging biofabrication and bioprinting strategies which use cellular aggregates and microtissues as 'building blocks' for the development of larger musculoskeletal tissues and/or organ precursors – a field of research that can potentially enable functional regeneration of damaged and diseased tissues. The application of cellular aggregates and microtissues for the engineering of musculoskeletal tissues, from vascularised bone to zonally organised articular cartilage, will be reviewed. In the context of engineering anatomically accurate tissues of scale, novel bioprinting modalities and their application in controlling the process by which cellular aggregates or microtissues self-organise is addressed, as well as key challenges facing this emerging field.

1. Introduction

The overarching goal of tissue engineering (TE) is the complete regeneration of a tissue or organ whose function has been impaired by injury or disease. Early failures in the field of TE has motivated increased interest in mimicking the natural processes

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that regulate cell fate and tissue development. This process, often termed developmental engineering [1,2], aims to recapitulate key features of the different stages of normal development as a means of engineering tissues or organs with biomimetic composition, structure and function. Developmental engineering looks to move away from some of the early concepts associated with the 'tissue engineering triad', which centred around the selection of cell type, soluble morphogens, and a scaffold substrate to control *in vitro* tissue formation. Here the quality of the engineered tissue ultimately depends on the aptness of these three selected components. However, due to their strong interdependence, altering one component of the triad commonly influences the effectiveness of the other components. Consequently, this approach tends to initiate a perpetual cycle of empirical attempts to find an adequate solution [1]. Instead, developmental engineering commonly focuses on the use of 'scaffold-free' systems, shifting the aim towards creating precursor tissues *in vitro* that are primed to follow a concerted progression *in vivo,* resulting in restoration through recapitulation of the target tissues native developmental programme.

Without a scaffold material, developmentally inspired TE seeks to leverage the cell's own ability to synthesis a biomimetic extracellular matrix (ECM) under the direction of appropriate exogenous cues – both soluble factors and biophysical signals generated by cell-cell and cell-ECM interactions. These biological processes typically involve cell condensation, proliferation, differentiation, matrix production and tissue maturation [3]. The principles of cellular self-assembly and self-organisation underpin scaffold-free approaches in TE. The former has been defined as a system in which order results from disorder in a spontaneous manner, without the introduction of external force or energy, resulting in a closed system [4]. In contrast, self-organisation can be defined as a process in which order appears as a result of external energy or forces being input into the system [4]. In the context of TE, the spontaneous arrangement of cells *via* cell-to-cell interactions as a means of minimising free energy is considered self-assembly. Typically, this process involves a non-adherent substrate, and a sequential set of phases that closely mirror native tissue formation. Self-organisation approaches include cell aggregate engineering where external forces generated by centrifugation is used to facilitate the aggregation of cells, and can involve processes like 3D bioprinting to impart direct cell positioning [3,5,6]. Tissue fusion is a collective term for a number of important events that occur during embryonic development to form organ structures [7]. It is the process by which two or more isolated cell populations make contact and adhere, resulting in the initially discrete 'fusing units' forming a union [7]. Whilst cell-cell contact is an important feature of both self-assembly and self-organisation, unlike self-assembly, self-organisation involves tissue fusion processes (cell-to-matrix contact, matrix-to-matrix contact and ECM remodelling). In the context of TE, during this time two previously separate cell populations within engineered microtissues are brought together to form a continuous neotissue [4]. Routinely, both approaches (self-assembly or self-organisation) result in a tissue that has gross morphological, structural similarities, and comparable functional properties to native tissue, by virtue of recreating fundamental processes that occur *in vivo* [4].

One field positioned to benefit from this paradigm shift is musculoskeletal TE. To date, complete *in vitro* biomimicry of musculoskeletal tissues, such as bone, cartilage and the osteochondral unit, has not been achieved using traditional TE methods. Given that the tissues of the musculoskeletal system rely heavily on function through form, the modest clinical impact of the field of musculoskeletal TE is perhaps unsurprising given its failure to recapitulate the structure and composition of native tissues. This review will focus on the use of cellular aggregates, microtissues and organoids as 'building blocks' for TE strategies [8], and specifically on how emerging biofabrication and bioprinting techniques are being used with such cellular structures to engineer tissues and organs of the musculoskeletal system. In this review, we define a 'cellular aggregate' as a body of undifferentiated stem or progenitor cells, while a 'microtissue' (also referred to as a 'spheroid') is defined as a body of cells that have been driven towards a specific phenotype *in vitro* by exposure to differentiating factors. As such, a microtissue exhibits a specific tissue phenotype and contains tissue specific ECM components. 'Organoids' are commonly generated from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) grown in 3D culture, were they self-organise into complex, functional and multi-layered tissues. As such, organoids are unrivalled in forming cellular and tissue arrangements analogous to those found in organs. Despite their great potential as tissue mimetics, organoids have limited sizes and reproducibility issues that limit their wide spread application in basic research and regenerative medicine [5,6]. However, because organoids can be produced in relatively large numbers they have the potential to be used as modular building blocks and/or embryonic seeds for scaled-up organ engineering [9]. The review will first describe how cellular aggregates, microtissues and organoids have been used for the engineering of bone, prevascularised constructs, cartilage, and osteochondral tissues. We will then explore how cellular aggregates, microtissues and organoids can be used as 'building blocks' within emerging biofabrication processes to engineer scaled-up constructs suitable for tissue engineering and regenerative medicine applications.

2. Cellular aggregates, microtissues and organoids for bone regeneration

Cell-based bone TE strategies can be broadly classified into those that attempt to recapitulate the process of either intramembranous or endochondral ossification. The latter approach, which involves the engineering of a cartilage soft callus *in vitro* for bone repair, can be considered a developmentally inspired TE strategy as it attempts to recapitulate key aspects of long bone development [10,11]. Remodelling of an engineered cartilage anlage has proven to be an effective method for recapitulating bone organogenesis ectopically [12,13]. However, generation of a similar functional bone organ, complete with marrow cavity, vasculature, and resident cell populations, at an orthotopic site is challenging, as is scaling such TE strategies to treat larger sized defects. This has motivated the development of TE strategies that seek to combine numerous cartilaginous microtissues together into a single construct primed for endochondral bone formation (Fig. 1A). This section will review the use of immature cellular aggregates, cartilage microtissues (sometimes referred to as 'callus organoids' [14]) and osteogenic microtissues (that recapitulate an intramembranous ossification pathway) for the regeneration of large bone defects.

2.1. Cartilage microtissues for bone tissue engineering

Pellet culture is a well-established method for chondrogenic differentiation of mesenchymal stromal/stem cells (MSCs) [15]. Recognising that cartilaginous tissues engineered using MSCs have an inherent tendency to proceed along an endochondral pathway [11,16], cartilage pellets have also been used as part of strategies to regenerate large bone defects [17]. Van de stok and colleagues implanted 6 cartilage pellets (2 mm – 3 mm ø), each containing 2×10^5 human MSCs (hMSCs), into 6 mm long femoral defects in rats. Pre-implantation, the chondrogenic pellets were rich in proteoglycans and collagen types II and type X, the latter indicative of a hypertrophic cartilage phenotype. The pellets promoted complete bridging of the defect, as early as 4 weeks post-implantation.

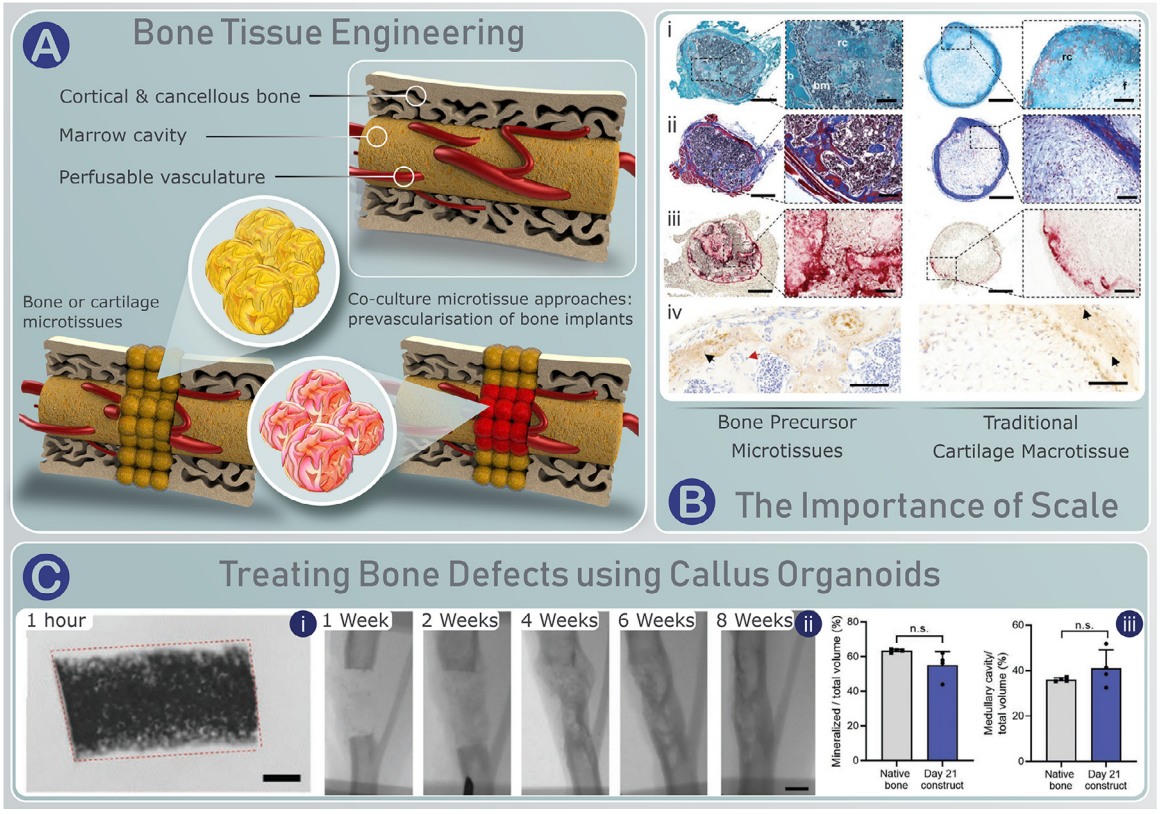

Fig. 1. A) Schematic representation of how microtissues can be utilised in bone tissue engineering. Bone precursor microtissues can be used alone, or in combination with vascular microtissues to generate prevascularised bone implants. B) The importance of scale when engineering cartilage precursors for endochondral bone tissue engineering. Histological comparison of bone precursor microtissues and a traditional cartilage macrotissue for bone tissue engineering *via* endochondral ossification, 4 weeks after ectopic implantation. i) Safranin O, ii) Masson's Trichrome, iii) tartrate-resistant acid phosphatase (TRAP), and iv) Human osteocalcin (hOCN) staining. Collectively, histological staining indicated that cells (positive hOCN staining) within the implanted bone precursor microtissues contributed towards the generation of a ossicle, with trabecular struts, active remodelling (positive TRAP staining for osteoclast activity), and bone marrow. In contrast, a traditional cartilage macrotissue only mineralised at the periphery of the implant and is filled with fibrous tissue, with little to no evidence of a bone marrow cavity. Scale bars = 500 μm (overview), and 100 μm (zoom & iv). C) The same precursor microtissues can be used to heal a murine, critically-sized long bone defect. i) Brightfield image 1 hour after assembling numerous microtissues into a single construct (Scale bar = 1 mm). ii) X-ray images of the tibia defect treated with the microtissue construct (i) healing over 8 weeks (Scale bar = 1 mm). iii) Comparing the native tibia to the healed defect 8 weeks after implantation using *ex vivo* nano-CT quantification of mineralised volume (% of total volume) and the medullary cavity (% of total volume), (unpaired *t*-test). Figures B & C were adapted from [14].

Once the defect had been filled with a mineralised tissue, remodelling of this *de novo* bone over the following 8 weeks restored the gross architecture of the native long bone (formation of a bony cortex and medullary canal). However, this result was heavily donor-dependant, occurring in only one of the three human cell donors investigated. The variation in healing capacity may derive from differences in the chondrogenic capacity of each donor. It appeared that the more chondrogenic the donor cells, *i.e.* the more cartilaginous ECM deposited *in vitro*, the better the bone healing observed *in vivo*, however further studies are required to validate such a conclusion. It remains unclear what level of chondrogenic differentiation and ECM deposition must be achieved *in vitro* to ensure predictable and robust endochondral bone formation *in vivo*.

2.2. The importance of cartilage microtissue scale for endochondral bone tissue engineering

A well-documented limitation of high-density cell systems and cell laden scaffolds is their susceptibility to inhomogeneous deposition of matrix components and core-necrosis, which can translate to implant failure *in vivo* [18,19]. A simple approach to address this challenge is to reduce the number of cells within a spheroid or microtissue, which has been shown to improve neotissue formation without the need for complex culture regimes incorporating bioreactors for perfusion or mechanical stimulation [20]. Interestingly, simply by reducing cell numbers within an aggregate, early chondrogenic differentiation can be initiated without soluble exogenous cues. Moreover, genes involved in the development of cartilage and endochondral ossification were strongly upregulated in lower cell number aggregates in the presence of transforming growth factor beta 1 (TGF-β_1). As a proof of concept, the authors compared traditional homogenous cell seeding of a scaffold to seeding with multiple cellular aggregates. Despite the total cell number being four times lower in the latter, the cartilaginous tissue formed within a collagen scaffold using cellular aggregates was more homogenous. Additionally, a necrotic core observed in the traditional approach was absent in the aggregate system [20].

Recent work by Nilsson Hall et al. [14] took the concept of downscaling spheroids a step further, reducing the cell number per aggregate to 250 cells to limit the size of the developing microtissue to <150 μm in an attempt to mitigate diffusion limitations of signalling molecules. The study leveraged emerging knowledge on the resident bone cell subpopulations that contribute greatest towards native callus formation during fracture healing. As such, they employed human-periosteum-derived cells (hPDCs), as opposed to bone marrow derived stem/stromal cells (bmMSCs) that are more commonly used for endochondral bone TE, for the formation of self-assembled cartilage microtissues. The authors used gene niches, identified from the native autonomous control of transitional zones in a developing growth plate, as a

metric for determining the developmental stage of their microtissues. The phenotype displayed within late pre-hypertrophic cartilage spheroids during their *in vitro* differentiation lead to their definition as 'callus organoids'. In the context of developing callus organoids, a selected panel of developmentally inspired markers served as a means of directly assessing tissue development during the *in vitro* culture. These efforts culminated in a marked improvement in the reliability of *in vivo* outcome and provided support for the hypothesis that diffusion related challenges, which hinder tissue maturation within larger bone tissue engineered constructs, can be avoided by downscaling the cartilage rudiments to microtissues. Specifically, more consistent and complete bone formation resulted from ectopic (subcutaneous) implantation of constructs assembled from multiple smaller microtissues compared with more traditional macro-scale, self-assembled constructs (Fig. 1B). This effective mineralisation of the cartilage rudiment and subsequent maturation into a bone organ ectopically was also reproducible within an orthotopic bone defect model, where the healing process mirrored that of normal fracture healing (Fig. 1C & 4C). Taken together, the results clearly demonstrated the potential of modular pre-cursor microtissues, or callus organoids, to self-organise into a larger, functional bone organ. Additionally, the results strongly support the putative increased regenerative capacity of some understudied adult skeletal stem cell populations. The study exemplifies the developmental engineering paradigm, and offers a blueprint for how the design process can be implemented effectively in TE. Despite their relative success, Nilsson Hall and colleagues [14] recognise the need to continue work towards scalability of microtissues and organoids. In alignment with our own views, they identified a need for automated systems capable of generating the vast number of microtissues/organoids needed to create centimetre scale grafts, the risk inadequate vascularisation poses to the success of the highly metabolically demanding self-organised tissues, as well as the central role bioprinting is poised to take in structuring multicomponent tissues and organs.

2.3. Immature MSC aggregates for bone tissue engineering

Implantation of immature hMSC aggregates (also termed 'condensations'), coupled with the pro-chondrogenic cue TGF-β_1, has been shown to promote the regeneration of critically-sized bone defects through formation of a cartilage intermediate *in vivo* [21]. Interestingly, bone regeneration was further enhanced by imparting some physiological load, *via* compliant fixation plates, onto the developing cartilage within the defect. Such mechanical loading was found to extend the cartilaginous phase of the endochondral ossification process, and was associated with improved repair and consistent bridging of the defect [21]. This work helps to establish the importance of mechanical cues in regulating the progression of developmentally inspired bone regeneration approaches, particularly when implanting 'unprimed' stem/stromal cell condensations within an orthotopic defect.

The regenerative potential of immature MSC condensations can also be improved through the addition of an engineered microvasculature. Rivron et al. identified hedgehog (Hh) proteins, specifically Sonic hedgehog (Shh), as predominant morphogenic factors that are reactivated during fracture healing and neovascularisation. Motivated by this observation they generated multicellular spheroids containing hMSCs and human umbilical vein endothelial cells (HUVECs), and exposed the aggregates to exogenous Shh protein which was found to induce the formation of patent microvascular lumen within the spheroid *in vitro* in a dose dependant manner. This engineered vasculature anastomosed with host vasculature and resulted in a significantly higher number of perfused vessels *in vivo*. Furthermore, this enhanced vascular network contributed towards the formation of mature bone tissue *in vivo* [22]. It is easy to envisage how such multicellular aggregates could be used as the building blocks to generate large engineered bone, complete with a perfusable vasculature. Section 3 of this review (see below) provides further details of how cellular aggregates and microtissues can be used to engineer such vascular networks within musculoskeletal tissues. Taken together, these results emphasise the importance of biomechanical or biochemical cues, as well as the use of supporting cells, to control the *in situ* development and therapeutic impact of constructs generated using cellular aggregates and spheroids.

2.4. Osteogenic microtissues for bone tissue engineering

The direct osteogenic differentiation of MSC aggregates represents a promising strategy for bone TE. However, cells may undergo rapid phenotype reversion upon the withdrawal of soluble factors [23]. This presents a significant challenge for bone TE, as in spite of significant *in vitro* priming, implanted MSCs could contribute minimally to the regeneration of a bone defect. The provision of relevant ECM proteins has been proposed as a means of providing continued instruction and sustaining, *in vivo*, the osteogenic phenotype imposed *in vitro* [23,24]. Such a cell-instructive ECM is generated *in vitro* within osteogenic spheroids or microtissues, thereby generating a feedback loop which maintains their osteogenic potential through cellular interactions with cell-secreted proteins. Murphy et al. provided experimental support for this hypothesis, linking the increased and persistent osteogenic phenotype of MSCs within a spheroidal or microtissue system, upon removal of soluble cues, to cell-ECM engagements mediated by integrins such as $\alpha_1\beta_2$ [24]. Given that osteo-induced MSC aggregates retain their differentiation markers better than single cells, a number of studies have investigated if such microtissues can act as a direct replacement for individual MSCs within biomaterial constructs for bone formation *in vivo* [25,26]. Furthermore, the angiogenic and osteogenic potential of such microtissues can be enhanced by hypoxic pre-treatment of the MSCs during their monolayer expansion. Such environmental culture conditions were shown to improve viability and upregulate vascular endothelial growth factor (VEGF) expression without inhibiting the osteogenic differentiation of the cells. Furthermore, these preconditioned osteogenic microtissues significantly improved healing within critically-sized bone defects when compared with those treated with individual MSCs [27].

3. Cellular aggregates and microtissues for engineering vascular networks

3.1. Supporting angiogenesis and strategies to prevascularise engineered tissues

Engineering a supporting vascular network is integral to the regeneration of all vascularised tissues and organs, including musculoskeletal tissues such as bone and muscle. Cellular aggregates have been used to indirectly support the process of angiogenesis during tissue regeneration, while vascular microtissues have been used to prevascularise engineered constructs prior to implantation. In the context of the former approach, MSC aggregates have been shown to be potent drivers of neovascularisation through paracrine secretion of angiogenic factors such as VEGF and hepatocyte growth factor (HGF) [28,29]. Additionally, cell aggregates have demonstrated improved cell survival rates in ischaemic environments due to the cells within the aggregate being naturally preconditioned to hypoxic conditions and the upregulation of hypoxia induced survival factors, such as hypoxia-inducible factor 1-alpha (HIF1α) and anti-apoptotic factors [28]. Improving the survival of grafted cells is an important feature in the context of scaled-up

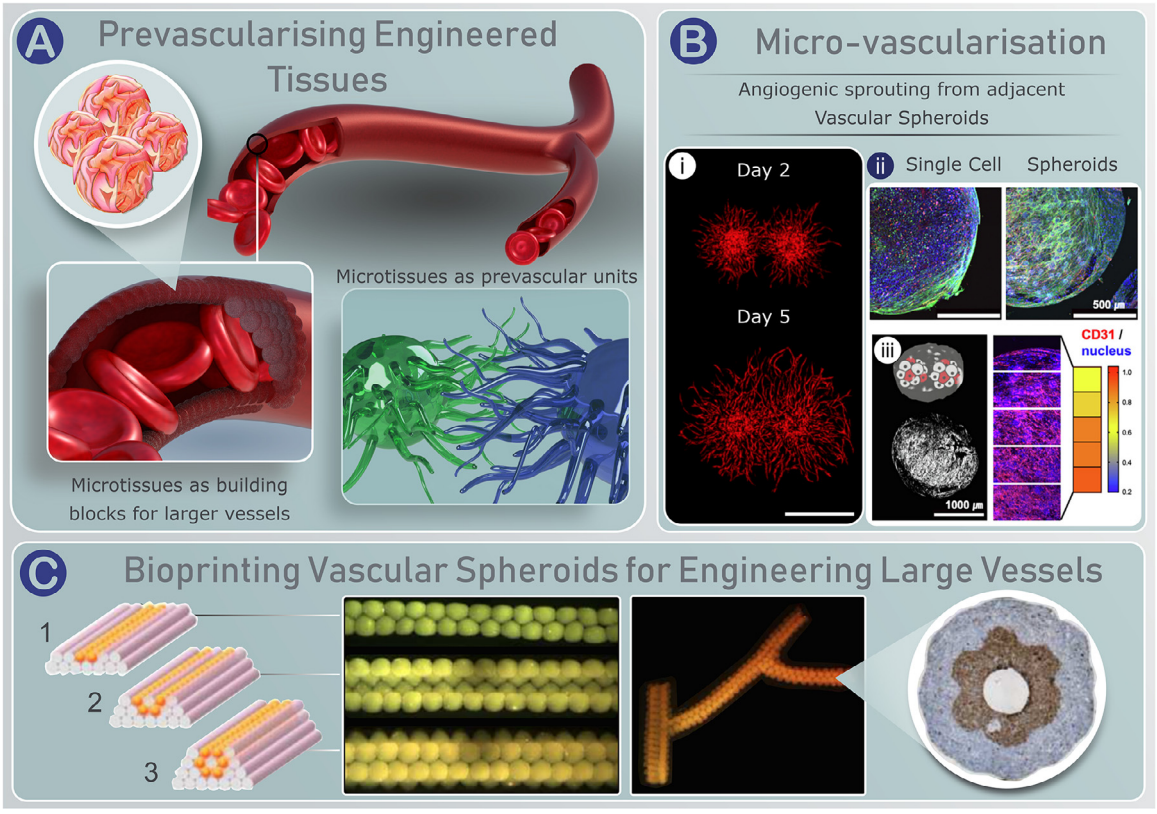

Fig. 2. A) Schematic of different approaches for prevasularising engineered tissues with blood vessels at different scales. The first uses vascular spheroids for engineering large blood vessels. The second employs spheroids as individual vascular units to form a prevascular network through endothelial sprouting. B) Vascular spheroids for prevascularisation. i) Adjacent HUVECs spheroids can sprout outwards and create a microvascular network (Scale bar = 1 mm) [34]. ii) MSC and HUVEC co-cultures can form vascular spheroids and be seeded within a hydrogel. Within this environment, these vascular units undergo angiogenic sprouting and form prevascular networks (iii) (Blue – DAPI, Green – f-actin, Red – CD31), Scale bar = 500 μm (ii) and 1 mm (iii) [33]. C) Spheroids can be bioprinted and self-organise into large, unified, and branched vessels. Bioprinting can be used to create tubular structures from spheroids (left-centre), it also allows to create connected bifurcations within the tubular structures (centre-right). Over time, initially discrete spheroids fuse to form a unified vascular tissue. Finally, bioprinting can spatially localise phenotypically distinct spheroid populations to form double-layered vascular walls. Here, spheroids expressing smooth muscle α-actin (brown) have been localised centrally within the bioprinted vessel (right) [47].

TE, where apoptosis due to oxygen deficiency within constructs in a physiological environment presents a significant challenge. The expression of these secreted angiogenic growth factors has been quoted as being 20- to 145- fold higher when compared to monolayer culture, and their effects so pronounced that conditioned media from spheroid culture is solely sufficient to accelerate wound healing [30].

Despite this potency, sprouting new blood vessels from the surrounding host vasculature and their subsequent ingrowth into the engineered tissue graft, is a complex, multifactorial process which requires significant time [31]. Consequently, for large grafts where implanted cells cannot survive solely by diffusion from the proximal host vasculature, prevascularisation of the engineered tissue or the inclusion of cellular aggregates with the potential to differentiate directly into vascular endothelial cells, represents a promising strategy for rapidly developing an adequate blood supply (Fig. 2A). This process accelerates vascularisation by anastomosing a microvascular network, typically preformed *in vitro*, with the immediate host vasculature through inosculation. Spheroids generated using cells derived from human adipose tissue have been shown to partially differentiate into vascular endothelial cells [29], as well as contributing directly to newly forming blood vessels within an implanted scaffold [29,32]. In a direct comparison with a traditional single cell approach, MSCs from spheroids were found to contribute twice as much to developing microvessels (~ 40% of cells in *de novo* vessels were identified as coming from the implanted spheroids, *vs* ~ 20% from single cell implants). Moreover, these developing vessels made a marked contribution to the overall vascularisation of the implanted scaffold by combining with invading host-vessels *via* inosculation [32]. Cellular aggregates generated using a co-culture of HUVECs and MSCs have also been shown to support the development of microvascular networks *in vitro* due to sprouting between individual aggregates (Fig. 2Bi & 4B) [33,34]. When encapsulated within a collagen/fibrin hydrogel, these co-culture spheroids have supported the formation of a more pervasive prevascular network compared to seeding co-cultures of monodispersed cells (Fig. 2Bi & ii). Additionally, this approach yielded a significant increase in the expression of osteogenic genes [33]. This suggests that the inclusion of vascular co-cultures can aid, not only in overcoming diffusion limitations through prevascularisation, but also enhance the differentiation of MSCs in applications where the target tissue is natively vascularised, such as bone. However, the mechanism of this synergistic interplay is currently undetermined and requires further investigation.

As previously discussed, successful examples of engineering tissues on a clinically relevant scale has been scarce. Generally, successful cases are limited to tissues with low metabolic demands, that are naturally hypoxic, are low in cell number, and are relatively thin (< 2 mm) and therefore at the limit of simple diffusion [35]. By extension, cellular aggregates or spheroids are high cell-density systems, and their size (diameter) is constrained by diffusion, with various 'maximum' diameters quoted throughout the literature. Aggregates/spheroids have been successfully prevascularised using HUVECs co-cultured with various cell types (bone marrow derived MSCs [36], human fibroblasts [37], human osteoblasts [38,39], human myofibroblasts [40]). As such, a strategy

of prevascularising each microtissue subunit is an attractive means of prevascularisation and potentially a route towards engineering organs with a rapidly perfusable vasculature. For example, it has been shown that prevascularised tissues of scale can be engineered by 'coating' multiple microtissues with HUVECs which migrate throughout the microtissues forming a prevascular network. Individually prevascularised microtissues can self-organise into a larger macrotissue, and unlike non-vascularised implants, connect with the host vascular network, resulting in the preformed capillary-like network being perfused with host erythrocytes [40]. In summation, these findings suggest that spheroids contribution's to vascularisation can go beyond their significant paracrine effects and reach into vasculogenesis, acting as individual vascular units capable of spontaneously developing new microvascular networks *in situ*.

3.2. Engineering larger vessels using microtissues

At present, the size of many vascularised engineered tissues is of the millimetre scale. As such, the microvascular networks previously discussed can be effective means of overcoming diffusion limitations associated with high cell-density systems. However, as such engineered tissues approach the centimetre scale (*i.e.* clinically relevant in humans), the need for a larger, immediately-perfusable vasculature increases. *In vitro* work with centimetre scale, functional tissues biofabricated from spheroidal building blocks has demonstrated that maintaining viability, and the consequent functionality of the engineered tissue, is only achievable with perfusable lumen within the body of the construct [41]. In light of this, engineering macroscale vessels, using vascular microtissues, which are amenable to integration within other spheroidal based musculoskeletal developmental engineering approaches is important for scalability (Fig. 2).

The principles of self-assembly and self-organisation have been used to engineer vascular constructs at a macroscopic scale. Gwyther et al. [42] have demonstrated a scalable method for generating vascular tubes from aggregates of smooth muscle cells. The technique involved seeding a high density cell suspension into a non-adherent annular well. After 48 h, the cells had spontaneously aggregated into a torus, contracting around the central post of the agarose well. The internal diameter of the rings (2, 4, and 6 mm) could be tuned by altering the size of the post, and the thickness of these tissues increased in accordance with the deposition of ECM components. Even without exogenous growth factors, individual rings were mechanically robust after only 8 days in culture, displaying mechanical properties that were favourable compared to traditional cell and hydrogel approaches. Furthermore, discrete rings could be manually aligned on a silicone mandrel, which fused together to form a continuous vascular tube ($>$ 2 mm in length). Despite this relative success, it should be noted that a cell line was employed in this study. The notable increase in proliferative capacity of a cell line compared to a primary cell source could account for the rapid generation of ECM components, tissue growth, and resultant mechanical properties reported. A more recent study employed a similar self-assembly method to form vascular tissue rings from human induced pluripotent stem cell (hiPSCs) derived smooth muscle cells [43].

When engineering functional tissues *via* prefabricated building blocks, it is important to consider the mechanical properties of the individual components. In the context of vascular tissue engineering, burst pressure is an important criteria. Achieving burst pressures approaching those of native vessels has come from fusing individual cell/tissue sheets [44,45]. Such cell/tissue sheets may be more suited to engineering functional vascular tissues than spheroids, because they can easily form continuous tubes, requiring only one fusion point. Several methods of self-organising cell sheets have been proposed. Some involve stacking sheets and, through the incorporation of HUVECs, permit prevascularisation of the developing 3D tissue [46]. Others have mimicked the concentric layers of native vessels, by rolling cells sheets comprised of distinct cells types [44]. Despite their inferior mechanical properties (*e.g.* burst strength), strategies relying on cellular aggregates or spheroids offer a distinct benefit over the aforementioned method as they allow the fabrication of branched macro-vascular structures. Norotte et al. [47] demonstrated that multicellular spheroids and cylinders can be used to biofabricate tubular structures as well as vessel-like structures with several bifurcations from a larger tubular structure (Fig. 2C). Efforts towards bioprinting microtissues for bone regeneration could benefit from this work, for example, by integrating such bioprinted vessels within a population of bone precursor microtissues.

4. Microtissues as building blocks for engineering articular cartilage

Numerous iterations and combinations of the three aforementioned elements of the 'tissue engineering triad' have been explored in attempts to engineer cartilage grafts mimicking the complex structure, composition and biomechanics of native articular cartilage [48]. These efforts have resulted in a number of different cell- (*e.g.* matrix-assisted autologous chondrocyte transplantation) and/or scaffold-based TE approaches reaching the clinic [49–51]. The relatively limited clinical success of many of these approaches has motivated further research in embryonic and postnatal developmental processes that can potentially be leveraged to improve cartilage TE [52]. Early developmentally inspired approaches utilised non-adherent surfaces to coax the self-assembly of articular chondrocytes and the production of a neocartilage *in vitro* [53]. Although scaffolds for cartilage engineering, arguably the most widely researched aspect of the traditional paradigm, can provide several benefits, they are not without their limitations [4]. As such, 'scaffold-free' approaches, unencumbered by the constraints imposed by a scaffold material or hydrogel, free the cells to self-assemble and self-organise (Fig. 3A). It is believed that through the generation of their own microenvironment, the limitations associated with scaffold-based TE (shielding from biophysical and biomechanical cues, toxicity and/or immunogenicity of the scaffold or degradation products, poor synchronisation between neotissue formation and material degradation, phenotype alteration, and restricted cell-cell communication [4,53]) can be circumvented. Additionally, the processes of self-assembly and self-organisation offer their own benefits for functional articular cartilage TE. These biological advantages centre on recreating keystone events that occur throughout the mesenchymal condensation during skeletogenesis, from the production of physiologically relevant cell adhesion proteins (neural cadherin (N-cadherin) and neural cell adhesion molecule (N–CAM)), to recapitulating specific and appropriate cell-ECM interactions and growth factor mediated signalling events associated with limb bud development [54]. As a result, such scaffold-free approaches can more effectively recreate the sequential events of mesenchymal condensation during limb bud development. Moreover, it is seen as a crucial step in reliably committing undifferentiated stem/progenitor cells towards a stable chondrogenic lineage *in vitro*, and the subsequent development of tissue which is biochemically and biomechanically comparable to that of early developing cartilage [54–56].

4.1. Lessons from cellular self-assembly for engineering stable cartilage

Considering the broader fields of self-assembly and self-organisation can assist in the development of high-quality engineered cartilage when using cellular aggregates and microtissues.

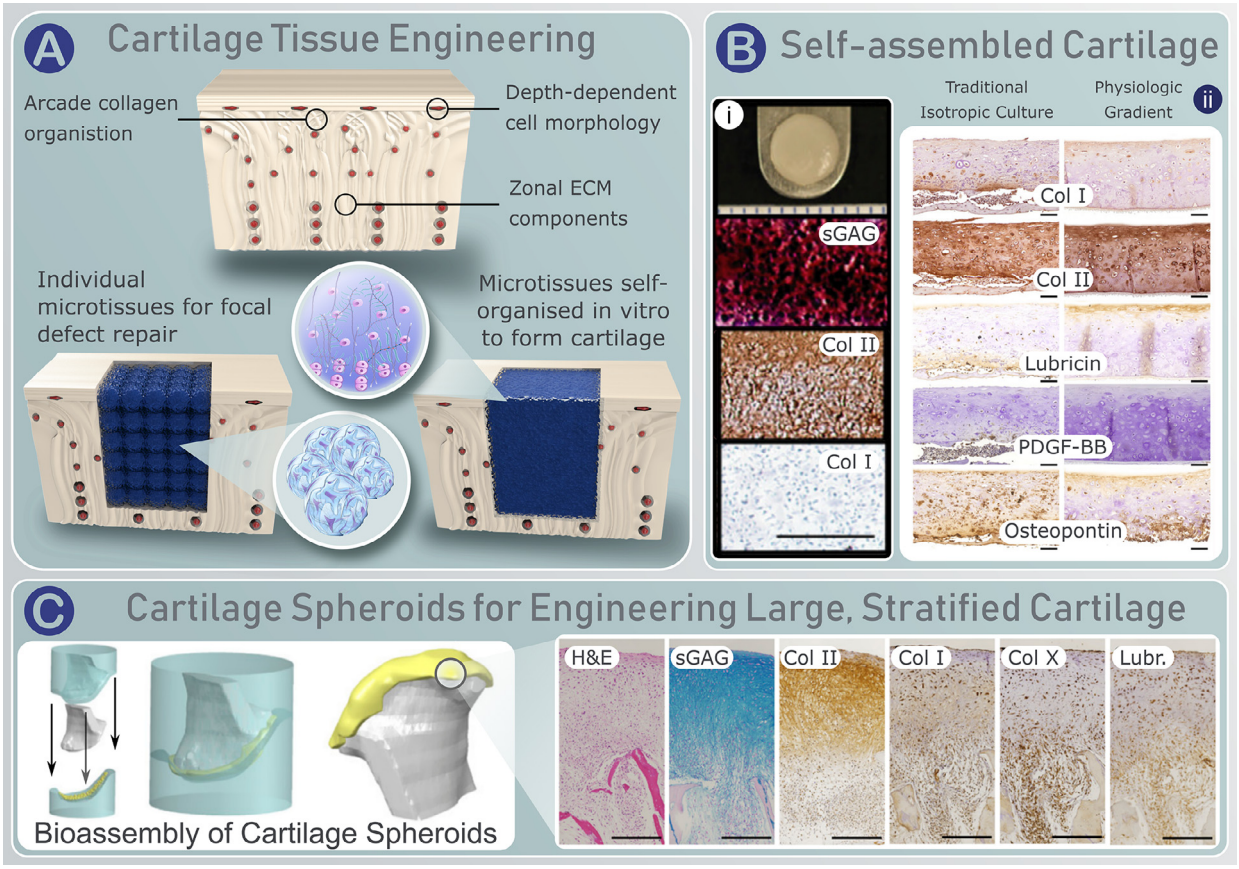

Fig. 3. A) Microtissues for cartilage tissue engineering. Individual cartilage microtissues can be employed directly within a defect, or allowed to first self-organise *in vitro* into a spatially organised tissue before implantation. B) Lessons from engineering a stable cartilage phenotype *via* self-assembly. i) Gross morphology and histology of self-assembled neocartilage treated with TGF-β and chondroitinase-ABC. Histologically, a stable, uniform and rich cartilage matrix is formed (positive staining for sGAG, collagen type II), scale bar = 200 μm [57]. ii) Recapitulating the spatiotemporal gradients present during native cartilage development have been shown as a means of spatially organising an engineered cartilage. Here, self-assembled human cartilage with physiologic organisation demonstrated more stability *in vivo* compared to a similar self-assembled cartilage that had been cultivated in traditional isotropic chondrogenic conditions. Histologically, the deposition of collagen type I, loss of collagen type II, absence of superficial lubricin expression, invasion of preosteoclasts/osteoclasts (Platelet-derived growth factor-BB (PDGF- BB)), and extensive osteopontin expression is indicative that cartilage cultured traditional isotropic culture conditions was more prone to undergo endochondral ossification *in vivo*, when compared to a more zonally organised engineered tissue (Scale bar = 100 μm) [58]. C) Cartilage spheroids can be fused to engineer large, anatomically-shaped and stratified cartilage. Cartilage spheroids were bioassembled on top of an anatomically shaped bone scaffold by pressing the scaffold into the spheroids using a two-piece silicone mould (left). After 5 weeks of chondrogenic cultivation, spheroids had fused and generated a stable cartilage tissue with zonal organisation (Scale bar = 500 μm) [68].

Several groups have successfully leveraged self-assembly *in vitro* to generate a highly bio-mimetic engineered cartilage (Fig. 3Bi) [57,58]. Additionally, others have demonstrated that MSCs which are self-assembled into discs or micromasses, as opposed to pellets used in traditional MSC chondrogenic assays [15], can elicit a more robust chondrogenic response [59–61]. Of particular note in this field is the work of Athanasiou and colleagues who have successfully used self-assembled cartilage discs as a platform to investigate the influence of growth factors [57,62,63], enzymatic treatment [57,63,64], and mechanical stimuli [65] on the development and maturation of scaffold-free cartilage tissue. Additionally, others have shown that spatiotemporally exposing self-assembled cartilage discs to physiologically inspired soluble cues can promote the generation of a spatially organised tissue [58]. Furthermore, this TE strategy supported the development of phenotypically stable tissues, where the *in vitro* engineered stratified tissue was resistant to uncontrolled endochondral ossification *in vivo*, thus retaining its physiological zonal organisation in a subcutaneous murine mode (Fig. 3Bii). As such, this work affirmed the putative benefit of recapitulating aspects of native cartilage development in the pursuit of maturing hMSCs into a stable and organised cartilage tissue. However, in spite of these promising findings, there are several challenges with translating such approaches to pre-clinical and human clinical applications [49,66]. For example, engineering anatomically defined tissues with comparable thickness to human articular cartilage is challenging using established self-assembly approaches. Fixation of these cartilage discs *in situ*, or their integration with an osseous phase with sufficient integration to withstand the substantial shear and compressive forces within a joint, presents a significant challenge. Complications during surgical fixation of scaffold-free cartilage have been discussed elsewhere [49]. Attempts to secure self-assembled neocartilage discs using fibrin glue with extensive joint immobilisation (6 weeks) have demonstrated significant graft failure (50% of implant remaining in place) after 24 weeks *in vivo* [67].

4.2. Engineering organised articular cartilage using microtissues

Using cellular aggregates or cartilage microtissues as building blocks may address many of the challenges of engineering anatomically defined cartilaginous tissues of scale suitable for human clinical use. For example, it has been demonstrated that MSC aggregates, cultured for 5 days in the presence of TGF-β_3 to generate cartilage microtissues, can fuse under external pressure to form a homogenous cartilage matrix [68]. In addition, the developing cartilage successfully integrated with an underlying bone scaffold in

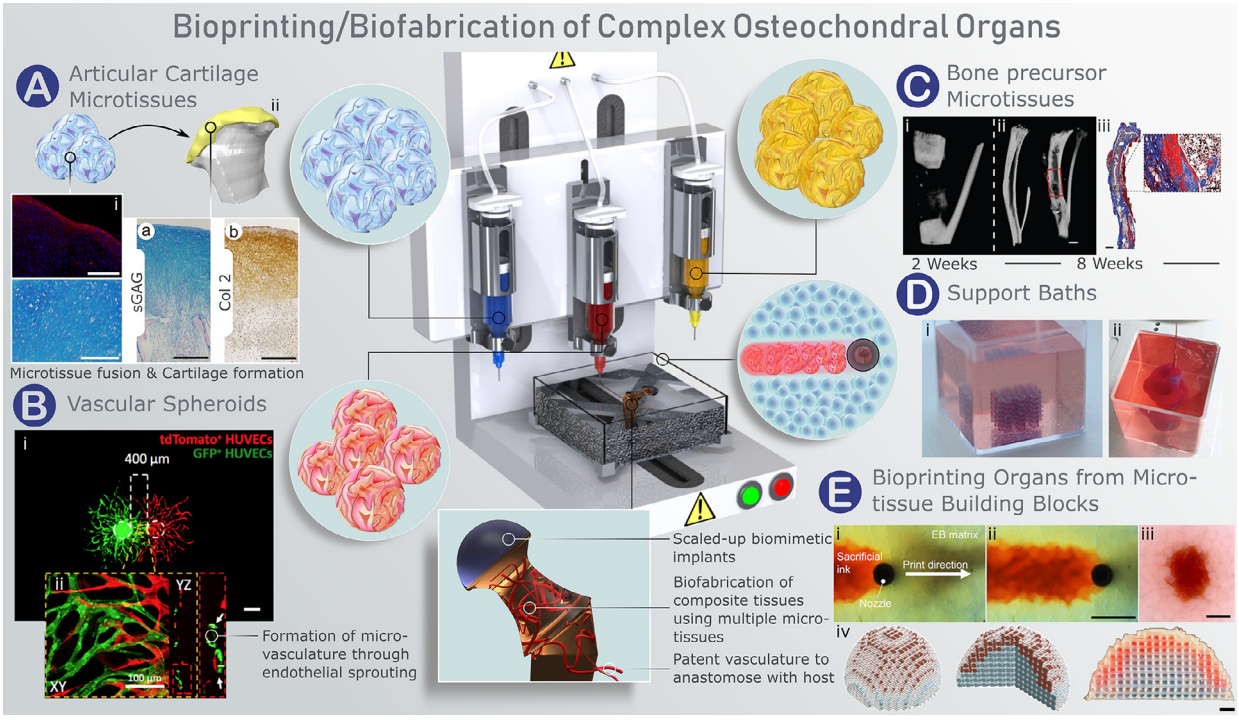

Fig. 4. Bioprinting/Biofabrication of complex osteochondral organs. A) Articular cartilage microtissues. i) Individual cartilage microtissues can fuse to form a homogenous cartilaginous tissue [68]. Positive tenascin deposition at the periphery of two fusing units (Red) indicates successful union between cartilage microtissues (Top), and homogenous sGAG deposition results from the successful self-organisation of the developing cartilage tissue (Bottom) (Scale bar = 200 µm). ii) Large, anatomically shaped cartilage can be engineered *via* fusion of cartilage microtissues. This engineered cartilage stains positively for canonical articular cartilage markers, sGAG (a) and collagen type II (b) (Scale bar = 500 µm). B) Vascular spheroids. i) Bioprinted HUVEC spheroids demonstrate angiogenic sprouting behaviour, forming capillary networks between adjacent spheroids after 7 days (Scale bar = 400 µm) (ii – High magnification at the interface region in the *XY* and *YZ* planes) [34]. C) Bone precursor microtissues. Implantation of developmentally inspired callus organoids into critically-sized bone defects enables the regeneration of the long bone with similar morphological properties of the native organ [14]. i) Nano-CT 3D rendered image at 2 weeks post implantation, ii) native bone (left) and treated tibia after 8 weeks (right) (scale bar = 1 mm). iii) Masson's Trichrome staining of the treated defect after 8 weeks indicating full bridging of the defect with cortical bone and the presence of a mature marrow cavity (Scale bars = 1 mm and 100 µm, overview and high-magnification respectively). D) Support Baths. i) A multi-layered woodpile structure printed within a support bath, demonstrating high print resolution and fidelity. ii) A heart printed within a support bath, complete with key anatomical structures such as, hollow left and right ventricles [94]. E) Bioprinting organs from microtissue building blocks. i-iii) SWIFT [41]. (i,ii)Time lapse of printing a sacrificial ink (red) *via* embedded 3D printing within a live matrix of embryonic bodies (EB) (Scale bar = 1 mm). iii) Cross-section of the channel imposed within the matrix of spheroids by the sacrificial ink, which following evacuation of the sacrificial material was perfusable (Scale bar = 500 µm). iv) Illustration of computer-aided design (CAD) models of the bioassembly of phenotypically distinct spheroid within a scaled-up hemispherical construct for osteochondral joint resurfacing (left). A proof-of concept image (right) where hydrogel microspheres, stained red and blue to represent chondrogenic and osteogenic phases of the osteochondral implant respectively, have been bioprinted within a polymer support framework (Scale bar = 2 mm) [71].

both a simple plug configuration as well as in a large, anatomically accurate implant. Similar to the tissue that forms in self-assembled discs, the study demonstrated that by mimicking some of the features present in the embryonic milieu, a cartilage, with physiologic stratification of matrix components (glycosaminoglycan (GAG), collagen type- II and X, lubricin), can be engineered by employing cartilage microtissues as building blocks (Fig. 3C & 4A). Furthermore, the engineered tissue possessed biomechanical properties (compressive modulus >800 kPA and friction co-efficient <0.3) approaching those of native cartilage after only 5 weeks of cultivation [68]. Limitations with this approach include its manual nature, requiring the external pressure of a polydimethylsiloxane (PDMS) mould to force the formation of a homogenous tissue. Given that spontaneous fusion of the cell aggregates was not achievable without these constraints, difficulties may arise when adopting this methodology to patient-specific geometries. Furthermore, it remains unclear if such approaches lead to the development of articular cartilage with native-like anisotropy in its collagen network. Others have mirrored these findings and have observed that an upregulation of numerous chondrogenic markers during cellular self-assembly does not cause a concomitant formation of a stratified collagen organisation [3,57,59]. Studies that have attempted to guide collagen organisation in self-assembled tissue have primarily utilised mechanical constraints or polymeric boundary conditions, demonstrating that improvements in collagen organisation correlate with more biomimetic compressive properties [69,70]. In the future it is possible to envision the use of automated 3D bioassembly strategies that enable the precise localisation of microtissues [71] within carefully designed 3D printed scaffolds that function to guide their fusion and growth, ultimately directing the spatial organisation of the engineered tissue. Here care must be taken to ensure that such guiding scaffold structures to not negatively interfere with the fusion and remodelling of the microtissues. Alternatively, microtissues could be combined with preformed collagen-based scaffolds that already emulate the arcade structure of articular cartilage. In one example of such an approach, collagen threads were woven in a zig-zag pattern to form tubular structures that mimic the collagen organisation in articular cartilage. Two compacted collagen sheets were then sandwich between these tubes, stabilising the configuration prior to cross-linking the collagen. The patent cylindrical pores (1 mm) that remained were populated with microtissues. The spheroids supported robust deposition of sGAGs and type II collagen, ultimately resulting in an increase in the apparent Young's modulus (by 60%) compared to the un-seeded scaffold. The construct demonstrated substantial elastic properties and a compressive modulus approaching that of naïve native articular cartilage [72]. Given the reliance native cartilage has on the spatial organisation of it macromolecular network,

finding an efficacious and reproducible approach for generating spatial organisation within self-assembled cartilage is essential.

4.3. The importance of cartilage microtissue scale for articular cartilage tissue engineering

The use of larger cartilaginous spheroids for cartilage TE have been associated with certain limitations, specifically an intrinsic heterogeneity in cell phenotype due to diffusional gradients within such tissues. These gradients can affect not only cell morphology, but also cause distinct regions of matrix deposition within a single spheroid. Specifically, the production of collagen I superficially, collagen I,II, and X in the middle regions of the aggregate, and an absence of matrix within the core due to apoptotic or dead cells have been reported [19]. As such, the validity of high cell density spheroidal models for cartilage regeneration have been questioned [19]. The formation of chemical gradients and suboptimal culture conditions in these larger aggregates can account for many of the aforementioned issues. Hence, the development of scaled-down versions of the progenitor aggregates is emerging as a logical alternative. To this end, studies have sought to create high-throughput methods for generating small (<200 μm) microtissues from a few hundred articular chondrocytes [73,74]. The archetypical single cell scaffold-based approach has been directly challenged using these microtissues. *In vitro*, the small (50 – 250 cells) microaggregates underwent a more pronounced downregulation of stemness markers and a subsequent nuclear translocation of Sox9 and upregulation of Sox9 associated genes compared to single cells. Furthermore ectopic implantation of un-primed micro-aggregates (no exposure to the chondrogenic morphogens such as TGF-β) elicited a more rapid and intense formation of cartilage when compared histologically to primed (+ TGF-β) single cell constructs. As might be expected, this difference was amplified if the microaggregates were also exposed to TGF-β during the short *in vitro* culture, forming cartilaginous microtissues [75]. However, given the potential negative impacts of using growth factors in therapeutics, the capacity to outperform traditional single cell approaches in the absence of exogenous factors emphasises the potent potential of aggregation techniques to steer stem cell fate both *in vitro* and *in vivo*. Additionally, by scaling-down cartilage microtissues, they can become compatible with current extrusion based bioprinting methods that have been used for the structuring of single-cell-laden hydrogels for several years [76].

To date, orthotopic implantation of such cartilage microtissues has been scarce. However, promising defect regeneration from larger spheroids treatments in the clinical environment (chondrosphere®) [3,77,78] suggests that the improvements seen *in vitro* by down-scaling spheroid size can lead to further improvements. Additionally, much of the work previously undertaken with self-assembled cartilage can be directly translated to applications where microtissues are intended as 'building blocks' for generating an engineered cartilage. All of these approaches have sought to improve the quality of the neotissue formed *in vitro*. Consequently, the addition of boundary conditions to drive collagen anisotropy [69,70,72], enzymatic treatment to re-balance the sGAG:Collagen ratio [57,64], ECM substrate materials [79], the inclusion of growth factor eluting biomaterial micro-spheres [80], or the use of cartilage progenitor cells [81–83], can all potentially be used to enhance the functional development of articular cartilage engineered using microtissues. Despite this, issues generally associated with cartilaginous spheroids, such as the aberrant cellular morphology and phenotype observed in the peripheral regions of such tissues, may impact the quality of the overall tissue generated when numerous microtissues are brought together to generate a single tissue of scale. With this in mind, and given the aforementioned challenges with achieving spontaneous fusion of adjacent cartilage spheroids [68,84], significant thought must be given in each case to ensure that the microtissues are fit for purpose. In summation, robust chondrogenesis is achievable by recapitulating the mesenchymal condensation observed during cartilage development. Furthermore, the organisation of this developing tissue can be modulated, at least in part, by the introduction of cues to guide the self-organising tissue. This provides encouragement that a mature, biomimetic cartilage tissue for the biological resurfacing of damaged and diseased joints is within reach using cartilage microtissues as biological building blocks.

5. Osteochondral tissue engineering

Self-organised progenitor cell aggregates have also been used to generate constructs to treat osteochondral defects [85–89]. In one such study, cell aggregates generated using adipose derived stem/stromal cells were fused to create cylindrical scaffold-free implants. These undifferentiated constructs were then used to treat osteochondral defects created in the patella-femoral groove of mini-pigs. Macroscopically, the treatment groups appeared to have filled with an abundance of cartilaginous tissue, whereas the tissue filling the empty defects appeared to be more fibrous and depressed from the defect surface. However, no significant differences in the quality of repair was observed between the control and experimental groups at either the 6- or 12-month time-points. Histologically, the repair tissue at the apical surface of the treated defect appeared filled with fibrocartilage and regeneration of the subchondral bone occurred *via* an endochondral pathway. In contrast, empty defects were entirely filled with fibrous tissue, with little to no regeneration of the osseous or chondral regions visible 12 months post-operatively [87]. Although microscopic scoring indicated a significant improvement in healing, there are several features of this early work worth noting. First, defects were created in a relatively low-load bearing site within the joint; it would be of interest to observed how such an immature, predominantly cellular, construct would respond to a more biomechanically demanding environment such as the femoral condyle. Additionally, although this treatment resulted in an improvement over the untreated control, this approach failed to consistently promote complete hyaline cartilage repair. Despite this, the study also raises interesting questions into the mechanisms by which the cells within the undifferentiated spheroids are guided by the surrounding environment, towards regenerating the distinct osteochondral tissues. Additionally, it provides encouragement that long-term regeneration of osteochondral tissues can be enhanced by the use of scaffold-free tissue engineering strategies, although direct comparisons with scaffold-based approaches are required to better understand the relative benefits of both.

6. Biofabrication strategies

As the platform technologies, hardware capabilities, and biomaterial science associated with bioprinting expands so does its scope. Traditionally, bioprinting involved the precise, user-defined spatial deposition of a single-cell suspension encapsulated within a bioink in three-dimensions. However, the remit of bioprinting is now extending towards the deposition of cellular aggregates, microtissues and organoids in hopes of bioprinting complex organ-units using these organoids as embryonic seeds [9]. The rapidly expanding toolbox available to bioprinting labs has pushed the boundaries of the 'biofabrication window' significantly from when print fidelity was predominantly defined by the shear thinning nature of the deposited cell-laden biomaterial (bioink) [90]. Martin et al. have recently proposed two prospective methods for 'organogenesis by design', which combine the

paradigms of self-organisation and bioprinting [9]. The first, leverages bioprinting to impose geometric design and sizes to direct a pool of printed stem/progenitor cells towards a developing organoid. When compared to traditional symmetrical, spherical self-assembly, the self-patterning of the bioprinted cells can potentially be guided through the user-defined micro-patterning. Alternately, bioprinting can be utilised in a bottom-up approach whereby numerous small organoid units, created prior to the printing process from stem/progenitor cells, are deposited in a spatiotemporal fashion to drive subsequent development. In this scheme, the patterning imposed during bioprinting is intended to instruct the formation of more complex, large-scale organogenesis from the smaller intermediate organ progenitor building blocks [9]. Within this section, an up-to-date insight into novel and emerging bioprinting/biofabrication strategies employed for the development of functional tissues and organs from high-density cell aggregates and microtissue precursors will be provided, with a focus on the technological advancements that have facilitated significant progress in the field.

6.1. Embedded bioprinting of cellular aggregates in supporting baths

Until recently, bioprinting millimetre scale structures with acceptable fidelity has been limited to polymer-rich bioinks [91]. However, the inherent opposing requirements of a bioink mean that these dense bioinks offer limited biomimicry and ultimately restrict tissue development. 3D bioprinting within a suspension media has emerged as a platform for patterning lower viscosity, biomimetic bioinks with excellent resolution and fidelity [91]. Microgel support baths have emerged as a means of supporting the deposition of both individual cells as well as cell spheroids [92,93]. These support baths, which act as liquid-like solids (LLS), are formed of granular hydrogel microgels tightly packed within cell culture medium [92]. Post-extrusion, the microgels provide physical support for single cells as well as cellular aggregates, whilst offering unrestricted diffusion of nutrients, waste, and other molecules by virtue of the interstitial spaces between the microgels and their high (>99% w/w) liquid composition [92]. This approach has demonstrated exceptional resolution and print fidelity, capable of reproducing personalised tissues and organs (Fig. 4D) [94]. Additionally, similar methods have been used to support both bone and cartilage by bioprinting cellular condensations and then maintaining them in long term culture within mechanically stable support medium [93]. In this study, human stem cells were the only component of the bioink. As such, they were able to coalesce *via* transmembrane proteins and differentiate along specific musculoskeletal lineages generating specific matrix components unhindered by an interstitial hydrogel. To achieve this, photoreactive groups were added to alginate micro-particles, which allowed the support bath to be cross-linked post-printing generating a mechanically stable substrate for extended culture.

6.2. Sacrificial writing into functional tissue (SWIFT)

The sacrificial writing into functional tissue (SWIFT) technique effectively inverts the aforementioned 'support bath' paradigm, whereby a sacrificial ink is printed into a slurry-support bath comprising predominantly of cellular spheroids [41]. As such, SWIFT demonstrates that by coupling a carefully selected ECM and 'organ building blocks' (OBBs), a support bath that displays strong shear-thinning behaviour with appropriate yielding ahead of the translating nozzle and self-healing in its wake, analogous to the microgels used in traditional embedded printing techniques is possible (Fig. 4Ei & ii). Interestingly, in the absence of densely packed OBBs, the ECM solution alone does not exhibit the necessary rheological properties to support embedded 3D printing. At physiological temperatures, the ECM undergoes marked stiffening, fixing the geometry imposed within the OBBs by the sacrificial ink as it is evacuated (Fig. 4Eiii). This methodology was leveraged to generate networks of perfusable tubular features embedded within the construct, which serve as template for vascular channels within the construct which could exceed 40 mm in length and 4 mm in thickness. In doing so, the authors successfully maintained cell viability within the extraordinarily dense (0.5 billion cells) living matrix *via* perfusion of hyper-oxygenated (95% O_2) culture media through the printed channels. Furthermore, attempts were made to endothelialise the lumen with HUVECs. Although a confluent layer of endothelial cells across the entire lumen was not achieved, the results suggest that HUVECs have the capacity to adhere to the fusing EBs and remain in place during perfusion. Patent channels were printed into various OBBs: compacted embryonic bodies (EBs), cerebral organoids, and cardiac spheroids, without disrupting the complex architectures present within the developing organoids [41].

6.3. Aspiration-assisted bioprinting (AAB) of spheroids

Although different support baths may provide an effective substrate for bioprinting microtissues as minimal units for organogenesis, the precise control over their positioning in the 3D space has presented significant challenge. The aspiration-assisted bioprinting (AAB) technique represents a promising approach to address this challenge [34]. In this method, spheroids are positioned using the minimal aspiration force (critical lifting pressure) to overcome gravity, buoyance force, hydraulic drag, and the thermodynamic barrier at the interface, whilst maintaining cell viability above 80%. Application of this approach has been demonstrated in both scaffold based and scaffold-free situations. AAB has also been investigated as a method for engineering osteochondral tissues. Retention of osteogenic and chondrogenic spheroid phenotypes was maintained following bioprinting and translated into fusion of the distinct spheroids with the histomorphological characteristics of an osteochondral interface [95].

6.4. Fluid-based singularisation

Given the complexity of engineering biological interfaces, such as the osteochondral unit, bioassembly of numerous different microtissues into a single implant capable of self-organising into an organ precursor will not be without its challenges. To this end, unique micro-fluidic systems combined with bioprinting methods are emerging as effective means of spatially organising spheroids for osteochondral tissue engineering applications. Advanced bioprinting methods have also yielded the biofabrication of a biphasic, hemi-spherical constructs intended for osteochondral resurfacing (Fig. 4Eiv). Here, a novel fluid-based singularisation module was employed to accurately insert individual spheroids (cell- or biomaterial-based) into the pores of a thermoplastic polymer scaffold. Automated deposition of the pre-differentiated microtissues or chondrocyte laden microgels did not impact long-term viability, fusion of adjacent μtissues, or cell phenotype, resulting in the formation of cartilage specific ECM proteins over 28 days of *in vitro* culture [71].

6.5. The kenzan method

Alternatively, spheroids can be spatially organised using the 'Kenzan' method. Here, pre-assembled spheroids are robotically aspirated and subsequently impaled on a micro-needle array, which serves as a temporary support. The distance between the needles (commonly 500 μm) is such that adjacent spheroids can interact

and secrete a supporting matrix [96]. In the absence of a supporting biomaterial, the Kenzan method has been shown as an effective method for assembling numerous spheroids to form live vascular tubes measuring 5 mm in diameter and 2 cm in height [97], cartilage constructs for the treatment of focal defects (3 mm Ø x 1 mm) [98] and cylindrical osteochondral implants measuring 5 mm Ø x 4 mm [88]. The latter aided in the regeneration (radiologically and histologically) of osteochondral defects in a pre-clinical animal model [88]. Despite this, several drawbacks unique to using the Kenzan method as a means of bioassembling spheroids have been identified. Predominantly, the inter-needle distance defines a relatively narrow range of spheroid diameters that will permit fusion and the development of a unified tissue. As such, it places an onus on generating optimal spheroids which could prove challenging when considering the use of unknown cell combinations, and/or culture conditions/periods [96].

6.6. Bioprinted-assisted tissue emergence (BATE)

Bioprinted-assisted tissue emergence, or BATE [6], aims to leverage bioprinting to accurately control the initial spatial organisation and densities of organoid-forming stem cells to permit their spontaneous self-organisation into larger, centimetre scale, tissues. Here, a custom-built extrusion-mode printer was fabricated by combining a syringe pump with an inverted microscope. The microscope stage controller was used to define the spatial positions, while the syringe pump allowed extrusion of cell/organoid suspensions *via* a pulled glass capillary. Cells were deposited into a bath of hydrogel precursor material which, after printing was crosslinked at 37 °C to maintain the spatial positioning of the deposited biologics.

The study successfully demonstrated that bioprinting is an effective method of controlling the spatial deposition of stem cells onto a bioactive ECM substrate whilst maintaining their ability to form complex structures reminiscent of those within a mature organ through self-organisation (such as lumens, branched vasculature and tubular intestinal epithelia with *in vivo*-like crypts and villus domains). Additionally, bioprinting permitted spatial and temporal control over the introduction of support cells, which can have a potent modulatory effect on developing organoids thus improving growth and development [6]. Although considerable precision is required from the bioprinting hardware, this approach does not require the development of an overly complex bioprinting modality. Instead, the bioprinter is used to accurately and precisely control experimental variables, such as cell density, initial tissue geometry and the proximity and positioning of co-deposited heterogeneous cell populations. As such, the influence these factors, and others (cell-matrix interactions and soluble factors), on the spontaneous cellular self-organisation into organoids can be robustly investigated. Interestingly, the development of the 'BATE' technique demonstrates that highly complex printing methodologies are not required to strictly define spatial control over organoid/spheroid deposition. Instead, identifying and engineering appropriate conditions between the developing organoids and the surrounding environment can allow the printed cells to create the geometrical complexity of the final tissue, through post-printing remodelling and self-organisation. As such, it seems bioprinting is best suited as a tool, within a design strategy, to help define initial conditions and creating a favourable environment for the naturally programmed organoid building blocks as well as their relevant support cells to self-organise a specific tissue or organ [6].

7. Outlook

Leveraging cellular self-organisation within musculoskeletal tissue engineering holds tremendous promise for generating constructs that mimic the native tissue's composition and structural organisation. This can be enabled by the use of novel biofabrication techniques that provide precise control over the location of cellular aggregates or microtissues in 3D space. In this paper we have summarised the many advantages of using aggregates, microtissues and organoids as building blocks for generating large, biomimetic musculoskeletal tissues. In common with other scaffold-free tissue engineering strategies, such approaches can promote the development of a more biomimetic tissue as the cells are free to interact and self-organise. Moreover, tissue engineering with microtissues is an inherently scalable process. This benefit comes from first engineering scaled-down microtissues that inherently overcome nutrient transport limitations and yield more consistent lineage commitment and ECM deposition. Once combined, these microtissues have exhibited the capacity to act as functional building-blocks for the biofabrication of larger tissues *via* self-organisation and remodelling into functional grafts.

Despite showing promise, some deficiencies with current strategies remain. Robust fusion between proximal microtissues can prove challenging, and failure to form a continuous tissue can compromise the effectiveness of the construct. Additionally, achieving significant tissue maturation *in vitro* may be unobtainable within reasonable culture times, and hence a truly 'scaffold-free' approach to engineering a mechanically functional musculoskeletal tissue may be unfeasible. Within the musculoskeletal system, tissues are often placed under significant physiological loads. In this review we have highlighted that modular assembly of microtissues alone may not be sufficient to generate a mechanically functional tissue, and reliance on a supporting scaffold material may still exist until *in situ* maturation has occurred [3,8]. Modular biofabrication approaches, such as the self-organisation of individual microtissues, alleviate, in part, the interdependence between tissue formation and scaffold properties that occurs *in vitro* as the biological components of an implant can be individually optimised prior to their combination with the selected scaffold. Interestingly, scaffolds and microstructural devices could provide a valuable platform to help direct developing microtissues and self-organising constructs [5]. If properly designed and implemented, a careful balance between engineered guidance and dynamic remodelling within developing tissues can be provided. Resulting instructive boundaries and degrees of freedom can yield control over self-organisation that better recapitulates that seen in embryonic development [5]. Implementation of successful tissue guidance could have significant impact for the engineering of spatially complex tissues such as articular cartilage, where controlling the architecture of the collagen network remains a key challenge in the field.

In addition to generating structured, mechanically robust tissues, another key challenge facing the biofabrication of large self-organised tissues is vascularisation. An adequate nutrient supply is a pre-requisite for the delivery of nutrients and oxygen to large-scale engineered tissues and organs. While several methods have attempted to form networks of small vessels/capillaries with the hope of mitigating diffusional limitations, generating a perfusable, large-scale vascularised network is yet to be realised *in vitro* [5]. The emerging bioprinting strategies outlined in this review and elsewhere are positioned to address some of the hurdles associated with scalability and vascularisation [99]. In particular, bioprinting offers a means of directing the process by which microtissues fuse and self-organise into structurally complex tissues. Additionally, it serves as a platform technology to allow the accurate combination of multiple different microtissue phenotypes, independently generated, in a single construct within the confines of a user-defined architecture. Such approaches could also be combined with other techniques such as cell sheet engineering, that have also been applied in orthopaedic tissue engineering [100].

The generation of anatomically accurate implants, such as large osteochondral grafts that comprise cartilaginous, bony, and vascular tissues, is achievable by leveraging recent developments in the fields of biofabrication and bioprinting. Microtissues are an appealing method for large-scale, automated biofabrication of human tissues and organs, but bioprinting must be carefully employed to direct their fusion and self-organisation in a programmable manner [99,101]. This will require further developments in bioprinting hardware to address the challenges outlined above and to ensure the successful clinical application of such bioprinted implants in regenerative medicine. Realising automated, high-throughput methods for forming vast numbers of microtissues with various phenotypes, bioassembly of these pre-cursors into continuous functional units capable of progressing along a specific (*e.g.* developmentally inspired) pathway, and creating *in vitro* conditions and external cues that promote the development of structurally organised and mechanically functional tissues will require sustained efforts and convergence between several disciplines. In addition, convergence of different biofabrication technologies will likely be required to achieve these ambitious goals and to enable the scalable, efficient and cost-effective engineering of such tissues and organs.

In conclusion, it is becoming increasingly apparent that following a developmental engineering process can facilitate the engineering of biomimetic tissues recapitulating key biological features at various scales. Using cellular aggregates, microtissues or organoids as biological building blocks within such bioprinting platforms will be central to future efforts in this field of research. Such bioprinted tissues are poised to transform the field of regenerative medicine in the decades ahead.

Declaration of Competing Interest

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