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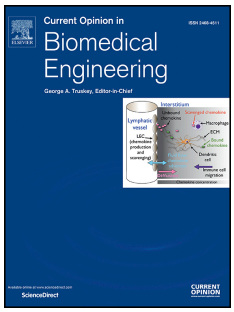

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Gagan K. Jalandhra, Kristopher A. Kilian

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Biofabrication approaches to fabricating gradients and interfaces in osteochondral tissue engineering

Gagan K. Jalandhra[a,c] and Kristopher A. Kilian[a,b,c,*]

[a]School of Materials Science and Engineering, UNSW Sydney, Sydney NSW 2052
[b]School of Chemistry, UNSW Sydney, Sydney NSW 2052
[c]Australian Centre for NanoMedicine, UNSW Sydney, Sydney NSW 2052

Abstract

Osteochondral tissue represents a complex biochemical and biophysical gradient between two distinctly different types of tissue. Its poor regeneration capabilities necessitate tissue engineering intervention; however, its complex structure and composition pose an immense engineering challenge. Though bone and cartilage engineering separately have seen success, fabricating the graded interface between these two dissimilar tissue types requires understanding and collaboration between multiple often-disunited disciplines. This review showcases innovative tissue engineering strategies utilised for fabrication of osteochondral interfaces in an attempt to bridge this gap, and highlights the potential of biofabrication techniques – namely 3D bioprinting – in providing a path towards future advancement in osteochondral and interfacial tissue engineering.

Keywords

Osteochondral; 3D bioprinting; biofabrication; interface; tissue engineering; gradient tissue

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1.0 Introduction

Osteochondral tissue encompasses the transition from stiff bone to soft articular cartilage at the end of long bones. It is vital in articular joint function as it aids in shock absorption, load distribution and stable motion [1-4]. Articular cartilage is avascular and aneural, making damage – from trauma, athletic injuries, pathological conditions or age-related degeneration – incredibly challenging to repair for the body [5, 6]. Inadequate regeneration leads to the wound site becoming necrotic, leaving a permanent defect [7]. Damage spreads to underlying bone and leads to degenerative joint disease and/or osteoarthritis [8]. The resulting loss in mobility is detrimental to patients' quality of life.

Over the last 15 years additive manufacturing techniques like 3D bioprinting have provided a paradigm shift in tissue engineering (TE) and regenerative medicine by allowing rapid prototyping of constructs for tissue repair. Traditional approaches involved inoculating pre-formed (bio)polymer or decellularized tissue scaffolds with cells, allowing little-to-no control over local features. 3D bioprinting enables greater spatiotemporal control of local scaffold compositions, mechanics, cell populations and biochemical localisation [9-11]. Though this has allowed greater biomimicry, recapitulation of complex tissue with functional attributes attained through biochemical and biophysical gradients, such as the osteochondral interface, remains a challenge.

Current osteochondral repair strategies yield negative side-effects and poor long-term success [12-14]. TE strategies require restoration of all elements comprising osteochondral tissue: bone, cartilage, and the interface [15]. Though separately bone and cartilage engineering have come far, osteochondral repair requires understanding and recapitulation of the complex interactions between the two distinctly different tissues. Several approaches have attempted to mimic the hard-to-soft gradient using bi- and multi-phasic biomaterials design [16-19]. Advancements in additive manufacturing techniques hold the key to improved osteochondral tissue repair.

1.1 Structure of the osteochondral interface

Bone and cartilage are structurally, mechanically, physiochemically, and biologically very different tissues. Osteochondral interfaces represents a smooth, continuous transition in structure, composition, and function. Bone extracellular matrix (ECM) consists primarily of collagen I fibrils, hydroxyapatite, and structural proteins, e.g., osteocalcin, osteopontin, bone sialoprotein and thrombospondin [20, 21]. It is highly vascularised. Cell populations present include mesenchymal stem cells (hMSCs), osteoblasts, osteoclasts, and endothelial cells [22, 23]. Subchondral bone and calcified cartilage have elastic moduli of 3.9 ± 1.5 GPa and 0.32 ± 0.25 GPa, respectively [24].

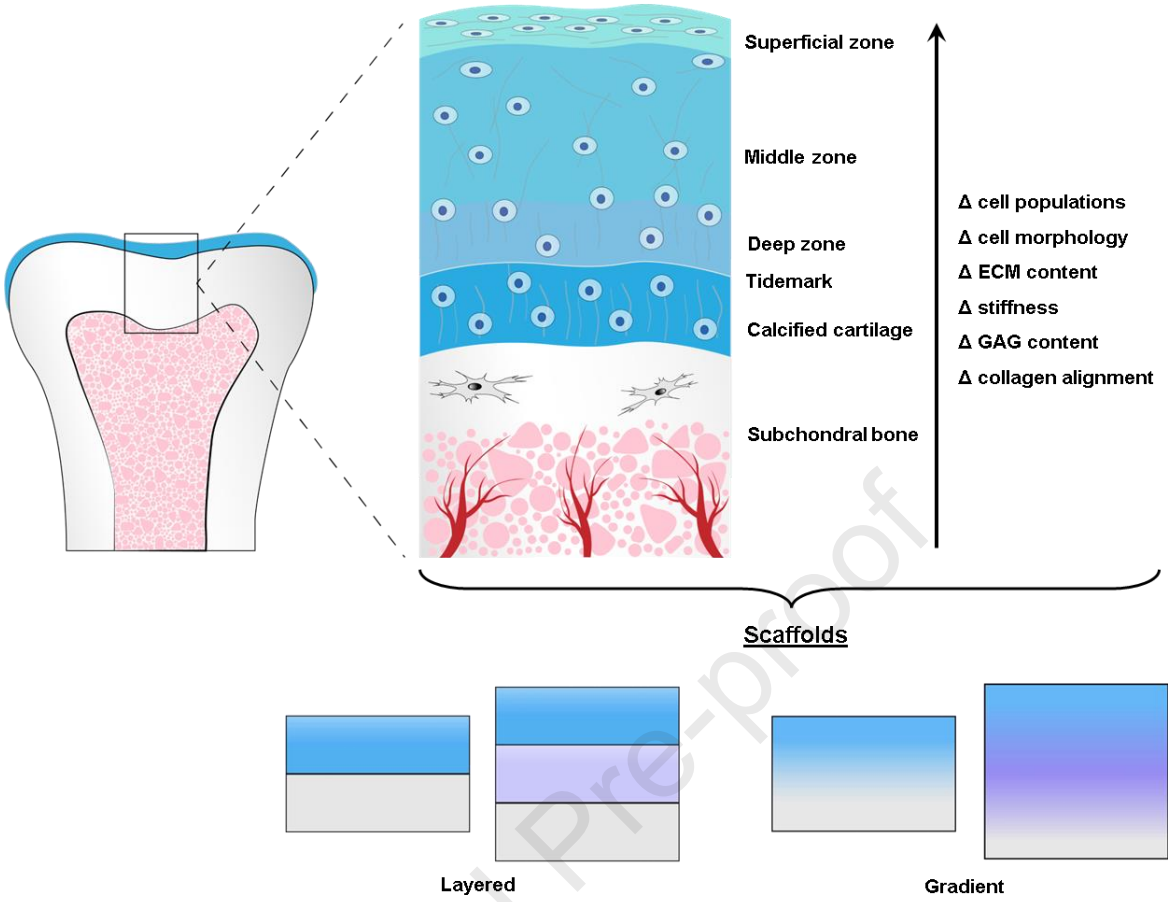

Figure 1: *Schematic showing the complex nature of the osteochondral interface (top) and tissue engineering approaches used to mimic the changing properties (bottom). Bone and cartilage contain very different properties and compositions, and engineering of the osteochondral interface requires understanding and recapitulation of complex biochemical and biomechanical interactions. Traditionally, layers of scaffolds promoting bone and cartilage formation were adhered together (bottom left), but recently there is a shift toward continuous graded scaffolds (bottom right).*

Hyaline cartilage comprises an ECM rich in collagen II and proteoglycans [17, 25]. Chondrocytes make up the entire cell population but only 2% of total tissue component. Collagen orientation changes with height, from more vertical near subchondral bone to more horizontal near the surface (**Figure 1**). This facilitates articulation and proper load transduction to the bone. Chondrocyte shape, size, density, and orientation also change with height, becoming increasingly populated and flattened closer to the surface [25, 26]. As chondrocyte populations change, so do their pericellular molecular environments. For example, hypertrophic chondrocytes at the surface produce a collagen X-rich matrix. Types of proteoglycans and glycosaminoglycans (GAGs), and their concentrations also change with height. High proteoglycan content near bone (deep zone) provides the greatest compressive resistance. Stiffness changes from 600 ± 50 kPa in the deep zone to 240 ± 50 kPa near the surface [27, 28]. Stiffness is reported to increase with loading frequency and amplitude, owing to cartilage's viscoelasticity [29]. Osteochondral tissue thus consists of several biochemical,

biomechanical, and cellular gradients which play key roles in its structure and function. These complex arrangements make it an immense challenge to recapitulate *in vitro*.

1.2 Current clinical repair strategies for osteochondral injury

Clinical strategies for cartilage or osteochondral repair include arthroplasty, microfracture, autografts, allografts, and autologous chondrocyte implantation. However, it is widely agreed that these yield inadequate outcomes [12-14]. Microfracture – involving debridement and subchondral drilling to stimulate bone marrow – causes donor site morbidity, lasting pain, and results in the formation of weaker, less viscoelastic fibrocartilage [18, 30]. As of 2016, the reported 5-year re-surgery rate for microfracture was an unacceptable 30-50% [31]. Mosaicplasty, wherein an autograft is taken from a non-load-bearing site, results in donor-site morbidity, and is limited by the size of graft that can be safely taken [32]. Allografts are limited by donor supply and risk disease transmission. Both allografts and autografts require topography matching and exhibit long-term failure due to poor integration [31, 33]. Though chondrocyte implantation has shown promise, it suffers from chondrocyte de-differentiation post-isolation and instability of cells at defect sites [34, 35]. Use of a scaffold, i.e., matrix-assisted cell implantation (MACI), overcomes this by ensuring chondrocytes remain at the defect site, however MACI scaffolds fail to address the different properties and gradient nature of bone and cartilage comprising osteochondral tissue [36].

1.2.1 To scaffold or not to scaffold

Bone is highly vascularised and has relatively high osteoclast and osteoblast populations which are able to easily degrade implanted scaffolds and deposit bone matrix. Contrastingly, cartilage is largely avascular and hypocellular, thus lacks the ability to readily degrade scaffolds [37]. It has been reported that presence of scaffolds at implantation sites over time can actually prevent hyaline cartilage regeneration and promote fibrocartilage growth [38]. Further, there are concerns surrounding immunogenicity and long-term effects of degradation products. This has led some to argue for development of scaffold-free approaches for chondrocyte or stem cell delivery to defects [37-39]. Though outside the focus of this review, it is important to acknowledge this perspective. Majority of literature agrees with requirement of a scaffold for osteochondral TE whilst acknowledging the necessity to understand and harness the body's natural regeneration capabilities [36, 40, 41].

Scaffolds are required to be highly porous to allow nutrient and metabolic waste transport, and accommodate cell infiltration [42, 43]. They should support cellular attachment, proliferation, and differentiation. Scaffold mechanical properties (e.g., stiffness) must match those of the tissue being modelled. If implanted, they should not elicit an adverse immunological response [44, 45]. For surgical applications, it is beneficial that scaffolds be injectable for minimally invasive application. They should be biocompatible, ideally with tunable degradation rate to match the cell or tissue growth [45].

1.3 Tissue engineering advancements in replicating osteochondral tissue

In the last 20 years, there has been a shift towards implementation of bi- or multi-phasic scaffolds for more accurate native osteochondral tissue recapitulation. Two or more phases are used – one promotes cartilage formation, and another promotes bone formation. In the early 2000s, this consisted of joining two or more separate layers via sutures or glue, resulting in abrupt soft-to-hard transitions [46-49]. These were problematic for numerous reasons including delamination over time, unwanted adhesive remnants, adhesives disrupting cell and nutrient movement, and sutures causing damage to wound sites. Additionally, they did not recapitulate the gradient nature of the interface which is essential for functional force transfer during mechanical loading.

Recently, there is a trend towards continuous bi- and multi-phasic gradient scaffolds containing graded physical or biochemical variations. Though decellularised osteochondral tissue provides excellent architectural mimicry, dependence on donors, harsh processing and limited modification flexibility render them relatively impractical [50]. Hydrogels overcome these and can provide greater control over features. Physical gradients are commonly achieved via stiffness variation of hydrogels, or incorporation of osteo-inductive and -conductive calcium phosphate micro-/nano-particles in graded concentrations (**Figure 2**) [51-58]. Chemical gradients are generally achieved via local release of osteo- and chondro-inductive factors bone morphogenic protein-2 (BMP-2) and transforming growth factor-β1 (TGF-β1) to drive osteochondral differentiation of stem cells. Commonly utilised scaffold materials include printable hydrogels such as alginate, collagen, gelatin methacryloyl (gelMA), polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(lactic-acid) (PLA) [55, 56, 59, 60]. Below we look at examples of scaffolds where local biophysical or biochemical properties are varied whilst maintaining matrix continuity.

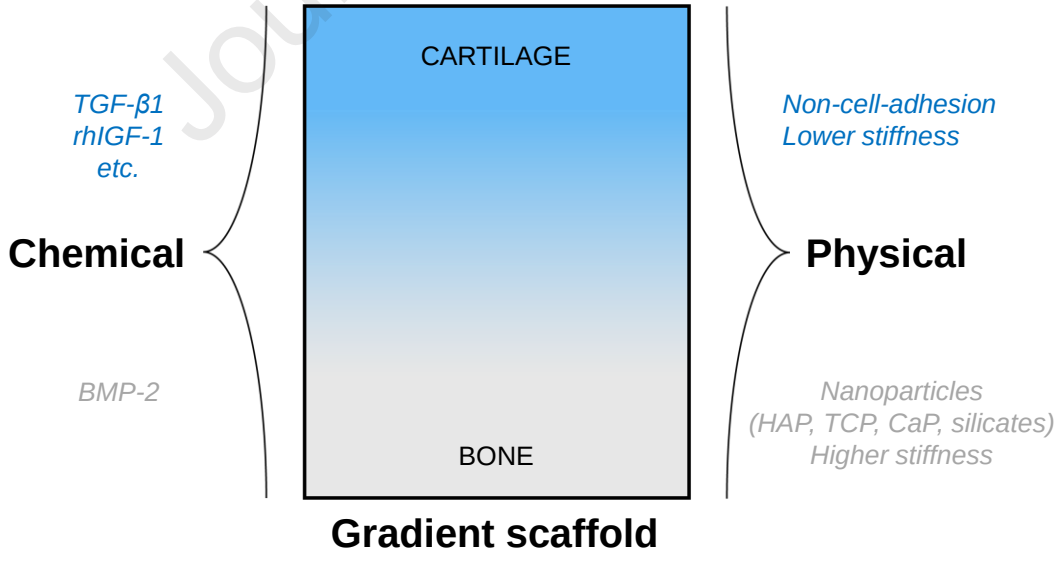

Figure 2: *Gradients in scaffolds can be formed via incorporation of chondrogenic and osteogenic biochemicals or by physically tuning hydrogel properties.*

1.3.1 Scaffolds with biophysical gradients

Physical gradients in osteochondral scaffolds are achieved via incorporation of inorganic nanoparticles (e.g. CaP, HAP, TCP). Bittner et al. employed multichannel extrusion 3D printing to fabricate porous PCL and PCL-hydroxyapatite (HAP) scaffolds with ceramic content gradients [54]. HAP nanoparticles (208 nm) were physically combined with PCL powder at 0, 15 and 30 wt%, then extruded layer-by-layer to fabricate mesh-like scaffolds with varying mineral content. Scaffold porosity was controlled by printing droplets of the same material between layers. MicroCT imaging verified mineral content gradient and uniaxial compression testing showed stiffness variations. Cell activity and tissue-integration capabilities were not explored. Similarly, Liu et al. formed collagen scaffolds with gradient-like nano-HAP distribution via *in situ* crystallisation of diffused Ca^{2+} and PO_4^{3-} ions [61]. Again, cell activity was not explored. These strategies are representative of a large portion of current literature for engineering gradient scaffolds for osteochondral TE.

In an alternative approach, Singh et al. fabricated silk fibroin scaffolds with a seamless interface of regions presenting different biophysical cues to laden cells [62]. Higher β-sheet content in silk fibroin fibres formed stiffer regions (40 kPa) more conducive to seeded osteocyte maturation, whereas lower β-sheet regions were less stiff and more amenable for seeded chondrocyte growth. Similarly, Cross et al. explored cell activity in gradient scaffolds fabricated from cell-adhesive gelMA and non-cell-adhesive methacrylated-kappa-carrageenan (MκCA) [63]. Equal amounts were pipetted into custom wells (10 mm length), then photo-crosslinked to form covalently crosslinked structures with passive gradients formed via electrostatic interactions between the two hydrogels. Gradients were tuned by varying volumes of each hydrogel and idle time before crosslinking. Encapsulated hMSCs were more spread in gelMA regions after three days indicating descent towards osteogenic lineage, while those in MκCA regions showed rounded morphology indicating chondrogenic potential. Nanosilicate addition enhanced rheological stability but negligibly influenced cell morphology. Physically combining hydrogels with varying bioactive inorganic component concentrations, stiffnesses or elicited cell-behaviour have been demonstrated as effective strategies in fabricating osteochondral scaffolds.

1.3.2 Scaffolds with biochemical gradients

An alternative approach for osteochondral TE is encapsulation and spatial release of biochemical factors which promote stem-cell chondrogenesis and osteogenesis. Gao et al. employed layer-by-layer printing using a copolymer hydrogel based on N-acryloyl glycinamide and N-[tris(hydroxymethyl)methyl] acrylamide [64]. TGF-β1 was encapsulated in the top layers in a gradient fashion for chondrogenesis, and β-TCP in the bottom layers for osteogenesis. Although separately TGF-β1 and β-TCP incorporation enhanced hBMSC chondrogenesis and osteogenesis, formation of an osteochondral gradient was not explored. Nevertheless, this demonstrates potential for spatial control of cell activity for osteochondral tissue formation.

Gurkan et al. bioprinted micro-droplets (~300 μm diameter) of MSCs and growth-factors encapsulated in photocurable gelMA for *in vitro* osteochondral tissue formation [65]. Gene expression analyses showed droplets containing BMP-2 directed MSCs towards an osteogenic lineage, while TGF-β1 droplets directed them towards a chondrogenic lineage within the same construct. The interface was formed via deposition of each type of droplet in a jagged zipper-

teeth formation (**Figure 3A**). Though fluorescent tagging showed formation of a chemical gradient, MSC behaviour at the interface was not explored.

Wang et al. generated biochemical gradients inside alginate and silk fibroin hydrogels via encapsulation of growth-factor loaded microspheres [66]. They encapsulated osteo-inductive BMP-2 and chondrogenesis-inductive insulin-like growth factor I (rhIGF-I) inside PLGA and silk fibroin microspheres to achieve linear gradients of the two growth factors to promote hMSC osteochondral differentiation *in vitro*. Though increasing BMP-2 concentration led to enhanced osteogenesis along the gradient, rhIGF-I alone was not found to influence chondrogenesis. Rather, it enhanced osteogenesis. Although unable to generate osteochondral interfaces, this study demonstrated potential for spatiotemporally controlled growth factor release.

In a hybrid approach, Castro et al. employed stereolithography printing to fabricate PEG-Da scaffolds with nano-HAP for directing osteogenesis and TGF-β1 for chondrogenesis [67]. Scaffolds consisted of 3 layers. 20% nano-HAP in the hydrogel for subchondral bone, 10% nano-HAP for calcified cartilage, and 10 $ng.mL^{-1}$ TGF-β1 in PLGA-nanocapsules for the cartilage. Though overall upregulation of chondrogenic and osteogenic genes was reported, gradient-like ECM deposition was not explored.

1.4 Biofabrication as a tool to control interfaces in osteochondral tissue

3D bioprinting allows rapid prototyping of 3D structures and has provided a paradigm shift in TE and regenerative medicine by helping overcome some of their greatest challenges. First, it allows unprecedented spatiotemporal control over scaffold architectures and cell populations, facilitating more accurate biomimicry. Second, processes can be largely automated, allowing scalability and potential to overcome donor shortages [68, 69]. Biofabrication may hold the key to TE advancements for improved osteochondral engineering.

3D bioprinting involves 3 basic steps: 1) Generation of a 3D CAD model (manually or via CT, MRI, X-ray, etc.) 2) Generation of slice-by-slice code (g-code) which communicates the desired result to the 3D printer. 3) Layer-by-layer fabrication of the structure. Any post-printing treatments are then carried out. Printing techniques are characterised based on how the fabrication is done. The following section looks at promising 3D bioprinting techniques for osteochondral TE.

1.4.1 Inkjet printing

Inkjet bioprinting (**Figure 3 A**) deposits precise, controllable droplets of bio ink which are assembled layer-by-layer to generate 3D structures [70]. It is subclassified into thermal or piezoelectric based on droplet generation mechanism. Inkjet printing's advantages include low cost, easy setup, and high speed [11, 71]. Disadvantages include frequent nozzle clogging, high shear and thermal stresses, and possible cell membrane disruption [11].

Development of bioinks with appropriately low shear moduli and adequate post-deposition stability without negative impacts of cell activity remains a challenge. Gao et al. reported inkjet bioprinting-based fabrication of poly(ethylene-glycol) (PEG)-based scaffolds for MSC osteogenesis and chondrogenesis [72]. PEG modified with acrylated RGD and matrix metalloproteinase-sensitive peptides enhanced osteogenesis and chondrogenesis. 222 layers, each 18 μm thick, were printed in under 4 minutes to generate cylindrical constructs 4 mm thick and wide. Bedell et al. evaluated printing capability of gelMA-HAMA and gelMA-β-TCP composites via inkjet, extrusion and digital light processing for osteochondral TE [60]. Although osteogenic and chondrogenic differentiation were observed, there was no real relationship between differentiation and the inclusions. Further, incorporation of β-TCP significantly impacted cell encapsulation efficiency and in all cases, composites required stabilisation with xanthan gum or nanocellulose fibres for printability. Though inkjet printing allows controlled high precision deposition, advances in compatible bioinks are required for osteochondral tissue fabrication.

Another droplet-based technique, microvalve printing, which combines a triaxial movable stage and multiple pneumatically-operated print-heads, has shown potential for osteochondral TE. Celik et al. used this technique to bioprint ADSC-spheroids transfected with microRNAs to induce differentiation – miR148b for osteogenesis, and miR-140 and miR-21 for chondrogenesis – to produce scaffold-free osteochondral interfaces [73]. However, this process is extremely difficult to scale up. Further, bioink compatibility and nozzle clogging remain major drawbacks [74].

1.4.2 Laser-assisted printing

Laser-assisted bioprinting (LAB) utilises (near-) UV wavelength lasers to deposit bioink droplets onto a substrate. Focused laser pulses stimulate a ribbon which consists of layers of an energy absorbing material, followed by a donor material, followed by the bio-ink. Upon irradiation with the laser, the energy absorbing material causes vaporises the donor layer, creating a high-pressure bubble that causes bioink droplets to be propelled towards a substrate [75, 76]. LAB achieves resolutions up to picometres. Unlike most techniques, it is performed without nozzles, eliminating shear stresses and clogging [71]. Preparation of the ribbon, however, is time-consuming and becomes increasingly complex with multiple cell lines. Further, the effects of the high energy laser on biological matter are not fully understood [71, 77]. Due to these drawbacks, to our knowledge this technique has not been utilized in fabrication of osteochondral tissue.

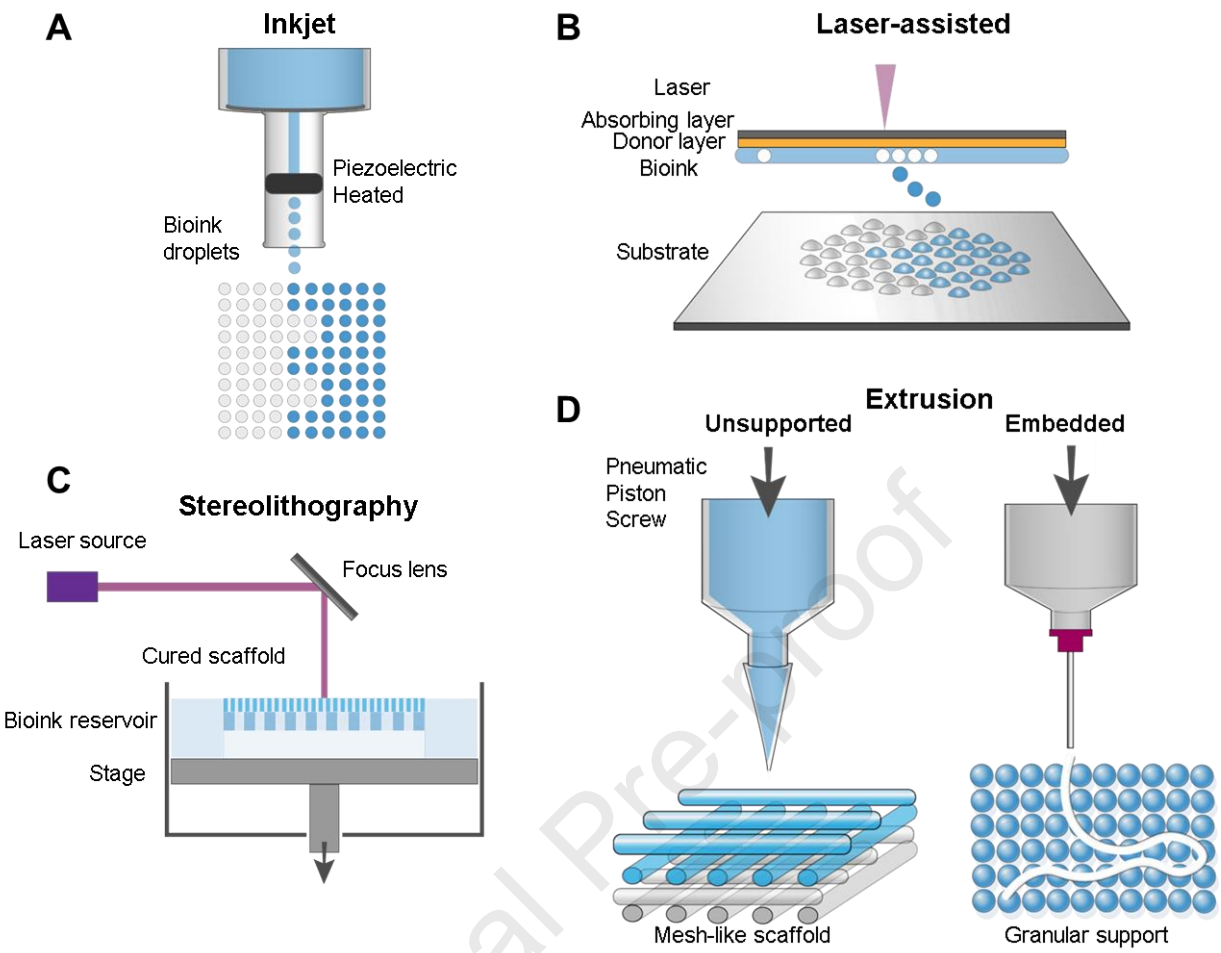

Figure 3: *Common 3D bioprinting techniques and example scaffolds generated. A) Inkjet printing is able to deposit droplets, which can be layered to generate 3D structures. B) Laser assisted bioprinting generated droplets with a high energy laser pulse and deposits them onto a substrate. C) Stereolithography locally cures bioink inside a reservoir. The stage moves vertically to generate 3D structures. D) Extrusion printing can either be unsupported, which is limited to mesh-like structures using log-like filaments, or embedded, which used a granular support to omnidirectionally deposit bioinks in complex geometries. Blue – bioinks for forming the cartilage phase in fabricated scaffolds. White/grey – bioinks for forming the bone phase in fabricated scaffolds.*

1.4.3 Stereolithography printing

Stereolithography-based printing techniques (**Figure 3 C**) utilise a focused light source (UV, infrared, laser) photo-crosslink material in a layer-by-layer fashion to yield a 3D structure [78]. It generates structures with high precision (1-2 µm), and is nozzle- and contact-free, so does not mechanically damage biological matter. Disadvantages include high setup costs, toxicity from photo-curing agents and inability to form horizontal gradients [79, 80].

Chen et al. demonstrated stereolithography for *in vivo* osteochondral defect repair in rabbits [81]. They formed implantable scaffolds with photocurable gelMA bioink supplemented with

MSC exosomes and cartilage ECM containing radially oriented channels. Analyses at 6 and 12 weeks showed enhanced chondrocyte migration and cartilage regeneration, along with formation of ossified subchondral bone tissue. Due to the nature of the technique however, scaffold geometry is limited to simple shapes, such as the cylindrical scaffolds in this study. Similarly, in the aforementioned study by Castro et al. scaffolds were limited to a mesh-like cylindrical structure [67]. Curing-laser penetration further limits construct height.

1.4.4 Extrusion bioprinting

Extrusion bioprinting encompasses techniques in which continuous bioink filaments are extruded pneumatically or mechanically (screw or piston) through a nozzle. It can be classified into two sub-categories: 1) supported – where the ink is extruded into a support matrix (granular hydrogel); and 2) unsupported – where the ink is extruded into air or an aqueous medium, supported only by itself.

Filament deposition has been utilised for fabrication of osteochondral scaffolds. Mesh-like structures with controllable porosity have been generated, with bio-inks containing encapsulated osteocytes and chondrocytes deposited in layer-wise fashion to recapitulate the bone-cartilage transition [82]. Similarly, varying concentrations of osteo-inductive calcium phosphate powders or other additives which drive osteogenic or chondrogenic phenotypes can be added to bioinks containing MSCs [51-58].

Syringe-based extrusion has successfully provided advancements in printing softer, low viscosity hydrogel bioinks. However, several rheological and chemical constraints of hydrogels disallow generation of 3D structures without loss of print fidelity when printing with no supports. Inks must be soft enough for extrusion without excessive shear forces – which may damage biological matter – but remain stable enough after printing to not distort under gravity [83-85]. Even with development of new bioink formulations, there remain inherent on geometries, such as inability to form overhanging structures, and reliance on high-viscosity inks. Scaffold geometries are limited to mesh architectures generated via layer-wise deposition of log-like filaments (**Figure 3 D)** [56, 57, 86]. To overcome limitations of extrusion techniques, musculoskeletal tissue engineers have shown increased interest in embedded extrusion printing wherein bioinks are extruded into support materials.

1.4.4.1 Embedded extrusion printing

Perhaps the most influential iteration of embedded extrusion printing is freeform reversible embedding of suspended hydrogels (FRESH) developed by Feinberg's group in 2015 [87]. It allows omnidirectional printing of soft tissue by deposition of hydrogel bioinks (collagen, alginate, etc.) inside a thermoreversible gelatin granular support bath which acts as a Bingham plastic during syringe-based printing. The support bath melts at 37°C, so can be removed while keeping cells alive, allowing fabrication of complex biological structures with resolutions of 200 μm. In 2019, FRESH2.0 demonstrated higher resolutions (200 – 20 μm) by reducing granular bath particle-size and polydispersity [88]. The technique has since been widely adopted and modified by the biofabrication community. For example, the Angelini group has

done considerable research on behaviour, modification and characterisation of granular support baths, including new materials and laden cell behaviour [89-91]. Jalandhra et. al omnidirectionally printed a ceramic bone-mimetic ink in granular suspensions optimised for chondrogenesis to spatially direct MSC osteochondral differentiation [92]. Various others have utilised this technique with bioinks of gelatin, collagen, alginate, agarose and cell spheroids [93-95]. Cidonio et al. printed laponite-gellan inks in agarose support baths for bone scaffold fabrication [95].

Being a nozzle-based technique, clogging is still a valid concern. Cell viability during longer printing times must be considered for generation of larger cellular constructs. The ability to print multiple bioinks at once, with isolated crosslinking mechanisms, inside biochemically and biomechanically heterogeneous support baths hold the key towards accurate recapitulation of complex native tissue.

1.5 Conclusion and outlook

Advancements in techniques which allow biofabrication of scaffolds with graded variations in biochemical and biomechanical properties have allowed more accurate biomimicry in osteochondral tissue engineering. Development of next generation biomaterials and utilisation of the full capacity of bioprinting techniques hold the key to recapitulating the complex structural and compositional heterogeneity of interfacial tissues. Key properties include compatibility with biofabrication techniques, the ability to manipulate local properties whilst maintaining matrix continuity to recapitulate the functional biomechanical and biochemical properties of native osteochondral tissue and practical scalability in order to be viable therapeutics. This requires a shift away from focusing on either bone or cartilage, but a deeper understanding of osteochondral tissue as a unit.

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☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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