Title: 3D bioprinting for musculoskeletal applications

Abstract

This review focuses on developments in the field of bioprinting for musculoskeletal tissue engineering, along with discussion on the various approaches for bone, cartilage and connective tissue fabrication. All approaches (cell-laden, cell-free and a combination of both) aim to obtain a complex, living tissues able to develop and mature, using the same fundamental technology. To date, co-printing of cell-laden and cell-free materials has been revealed to be the most promising approach for musculoskeletal applications because materials with good bioactivity and good mechanical strength can be combined within the same constructs. Bioprinting for musculoskeletal applications is a developing field, and detailed discussion on the current challenges and future perspectives is also presented in this review.

1. Introduction

Every 30 seconds, a patient dies from a condition that could be treated with organ replacement [1]. Organ transplantation has potential to be an efficient solution but is restricted due limited donor availability. Furthermore, organ transplantation requires complex surgical interventions and can lead to complications, such as organ dysfunction or rejection. Successful translation of tissue engineering and regenerative medicine research is key to alleviating the challenges in organ transplantation, but can also be applied to disease modelling and drug discovery (*Figure 1*). More specifically, improved understanding of the biological architecture and natural repair processes in adult tissues could aid the challenging fabrication of de novo organs, for these applications. For cells to self-assemble into tissues, they need an environment in which cells can remain viable and are able to adhere and migrate. The most important factors to consider are growth factors, nutrients, adhesion molecules, cells, materials and the technologies applied to enhance the fabrication process [2]. This review focuses on developments in bioprinting for musculoskeletal tissue engineering, and provides discussion on the various approaches for bone, cartilage and connective tissue fabrication, along with current challenges and future perspectives.
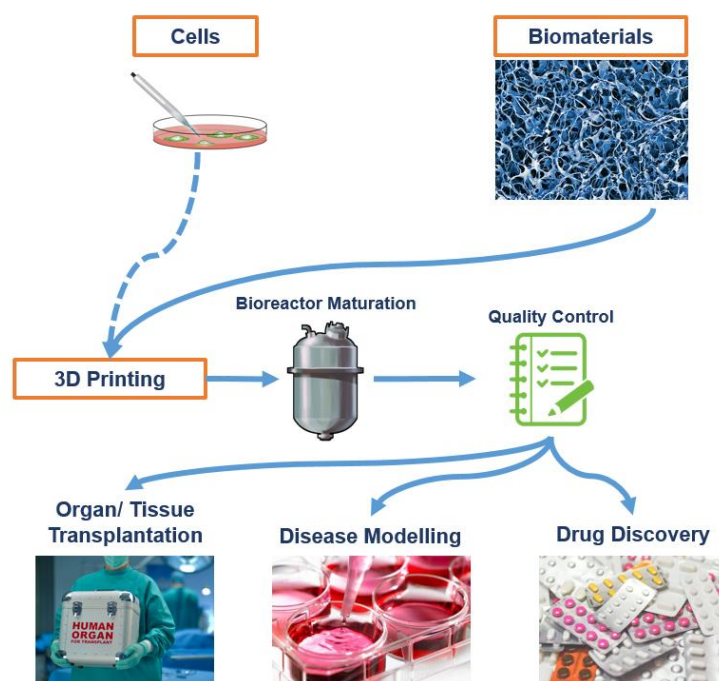
Figure 1. Translating tissue engineering and regenerative medicine research into healthcare applications for the future, including organ/ tissue transplantation, disease modelling and drug discovery.

## 2. Bioprinting and its role in musculoskeletal tissue fabrication

The musculoskeletal system (MSK) provides structural support for the body and comprises of vital tissue components such as bone, cartilage, muscles, tendons and ligaments. When these tissues are damaged through injury, their repair remains challenging due to their limited regenerative potential.

Every year, over 2 million bone grafts are performed worldwide, due to diseases, sarcomas, or trauma injuries [3]. In the US, musculoskeletal injuries reach 32 million per year, of which, 45% are represented by tendon, ligament, and joint capsular injuries [4]. Autografts are considered as the gold standard procedure for treating small MSK defects due to their compatibility with patients. However, obtaining these grafts causes defects in secondary sites and can delay patient recovery time. Furthermore, autografts contribute additional trauma injury and are limited by size.

For bigger defects, allogenic or biomaterial grafts are used. However, the use of allogenic grafts has several disadvantages including: immune rejection, the necessity for multiple surgical procedures to remove donor material, cost and limited tissue regeneration and revascularisation potential [5]. Synthetic grafts can also inhibit vascularisation and de novo tissue formation [6]. Tissue engineering advances can provide solutions to these problems. Nevertheless, traditional tissue engineering approaches are slow and cannot be used for large scale production of biological matter with the required complexity.

Bioprinting promises fast, on demand, and automated manufacturing of high resolution constructs. The process involves the use of 3D printing technologies to deposit cells or biological factors into predefined shapes and sizes [7]. Bioprinting permits stringent control on placement of cells within matrices and enables the arrangement of biological materials within composite, hierarchical structures and, patterns. This promises new opportunities to fabricate reproducible, patient-specific grafts with low risk of immune rejection. The most popular and promising bioprinting techniques include inkjet and extrusion printing. However, laser-assisted technologies are also in development [7]. Details on the various 3D bioprinting techniques are reported elsewhere [8].

To date, several research groups have bioprinted materials and cells for musculoskeletal applications. The two most common approaches used for 3D bioprinting, include the printing of cell-free and cell-laden materials. **Tables 1** and **2** outline how bone, cartilage, muscle, tendon and ligament tissues have been fabricated using these approaches. **Figure 2**, summarises the benefits of each approach.
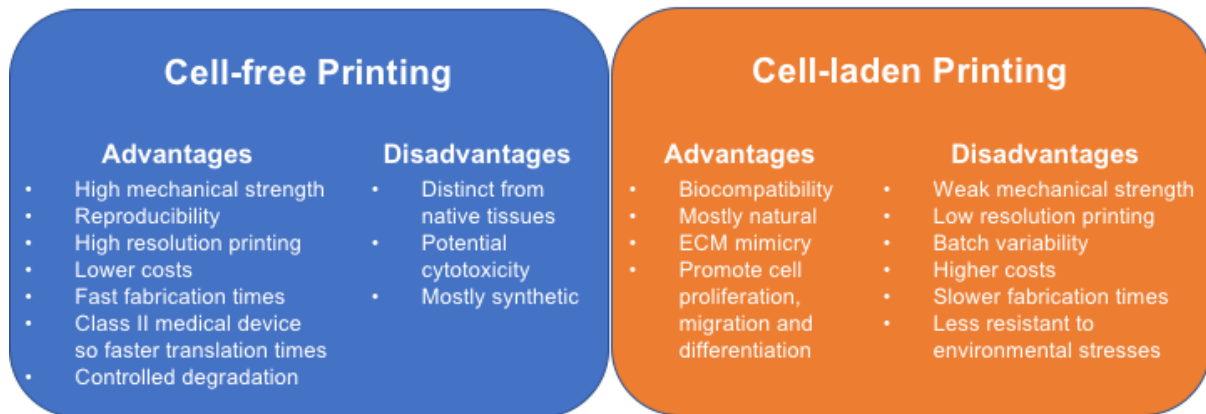
Figure 2. 3D printing approaches for musculoskeletal tissue fabrication. The advantages and disadvantages of cell-free or cell-laden 3D printing.

The current literature suggests that synthetic materials are more widespread for cell-free printing, while natural polymers have been commonly combined with cells, prior to extrusion. To evaluate the most popular approaches and materials used for bioprinting of musculoskeletal tissues, we reviewed the literature published in this area during the last 15 years. **Figure 3** summarises the various materials used for 3D printing using cell-free and cell-laden approaches in MSK tissue engineering. The data suggests that around 84% of the materials used for cell-free printing are synthetic. This is primarily because these materials provide the strong mechanical properties required for musculoskeletal applications. The remaining 16% of articles show feasibility of this cell-free approach using natural materials (such as collagen, alginate) and this can be due to their higher biocompatibility compared to synthetic materials.

One good example of cell-free printing for bone repair focussed on composite scaffold printing, where PCL-hydroxyapatite-carbon nanotubes were printed with pores in the range of 450–700 µm **(Figure 4)** [9][*]. Results show that a 4 MPa compressive strength was obtained and this is analogous to trabecular bone. The composite scaffolds also exhibited enhanced cell adhesion and improved hydroxyapatite bioactivity, when seeded with MG63 osteoblast-like cells. Nevertheless, the materials were distinct from native bone due to the presence of PCL and

carbon nanotubes, and the cells were seeded in a traditional engineering approach. This led to uneven cell seeding/ distribution.
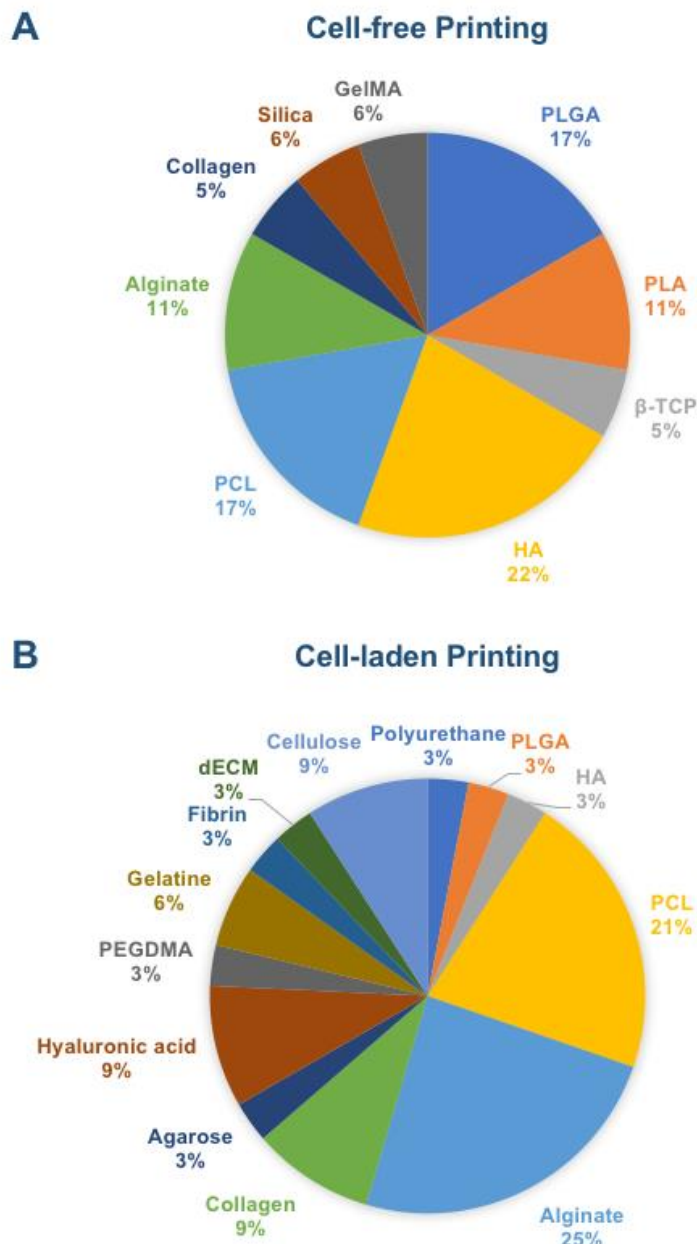
Figure 3. Percentage of various materials used in A) cell-free and (B) cell-laden printing for musculoskeletal applications. The data is based on articles published in the last 15 years using the search terms "3D printing", "bioprinting" and "bioink" associated with "bone", "cartilage", "osteochondral", "muscle", "tendon" and "ligament".

In the literature, naturally-derived biomaterials such as alginate [10,11], collagen [11], gelatine and fibrin [12], have successfully promoted cell adhesion, proliferation and osteochondral differentiation with both cell-free and cell-laden printing approaches. However, natural

materials were the most common materials used for cell-laden printing (70% of total reported in literature, Fig 3B) for musculoskeletal applications. This is predominantly because of their capacity to form gels that can support cell encapsulation and survival during the 3D printing process.
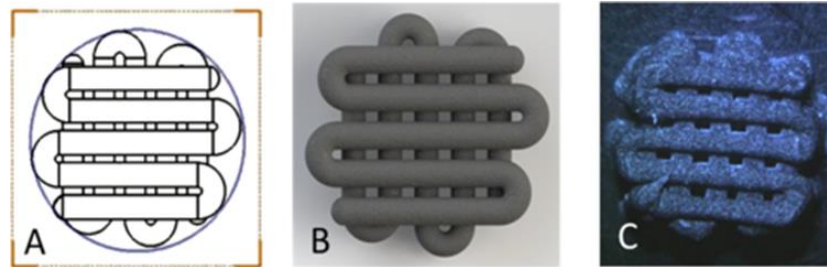

Figure 4. Structural properties of PCL-hydroxyapatite composites strengthened with carbon nanotubes for bone repair. (A) Technical drawing, (B) 3D simulation and (C) printed 3D scaffold with square pores. Image adapted with permission from [9].

Due to their poor mechanical properties, some of these natural biomaterials have been co-printed with other synthetic polymers for musculoskeletal applications. This can be realised by using multi-tool printing, which requires special modifications to printers, such as incorporation of additional print heads or extruders. Daly et al. used multi-tool printing to produce a mechanically reinforced cartilaginous template mimicking the geometry of a vertebral disk [13]. In this approach developmental precursors to an adult organ were bioprinted and the engineered construct functioned as a template for subsequent organogenesis in vivo. This was achieved by printing a PCL template, followed by the deposition of a RGD-alginate hydrogel laden with adult stem cells, as shown in **Figure 5**. When implanted into a mouse model, the resultant bioprinted construct supported the development of vascularised bone containing trabecular-like endochondral bone with a supporting marrow structure.
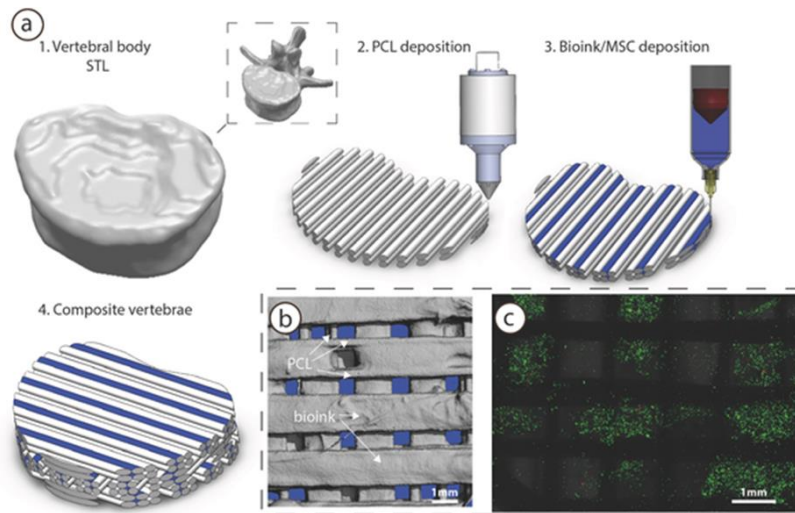
In another example using multi-tool printing, the vascularised bone was engineered when PLA was deposited with FDM printing technology, while GelMA containing BMP and VEGF was co-printed using SLA [14]**. This study demonstrated that the dual 3D printed constructs provided a hierarchically biomimetic bone-like structure, with multiphasic characteristics and potential for vascularised bone regeneration, as shown in **Figure 6**. This is a noteworthy approach to produce complex tissue structures in the lab. However, it is important to stringently assess the functionality of this vascularised bone and make quantitative comparisons with native tissues.
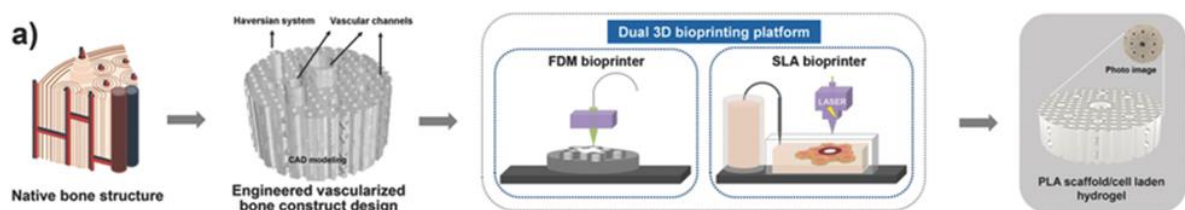
In addition to this work, interesting results have been obtained using synthetic materials. Poly (ethylene glycol) dimethyl acrylate (PEG-GelMA) was successfully inkjet printed with human chondrocytes for the repair of small osteochondral defects [15]. In another study, bone and cartilage tissues were fabricated through inkjet printing of human mesenchymal stem cells and, simultaneous deposition and photo-crosslinking of PEG-GelMA [16]. Others have achieved minimal print-head clogging by printing acrylate peptides and PEG hydrogels with human mesenchymal stem cells to promote robust bone and cartilage formation [17]. Porous bioactive glass/ alginate composite scaffolds have also been fabricated for bone tissue engineering using 3D printing [18].

As a prospective treatment for cartilage lesions, recent study reported use of 3D bioprinting approach to form cartilage mimetics using a nano-fibrillated cellulose and alginate-based composite bioink seeded with human-derived induced pluripotent stem cells (iPSCs) and human chondrocytes [19]. The bioprinted constructs could maintain pluripotency initially, and after five weeks, hyaline-like cartilaginous tissue with collagen type II expression, lacking tumorigenic OCT4 expression was observed. Furthermore, a significant increase in cell number within the cartilaginous tissue was detected. This study combines 3D printing and stem cell technology to generate viable tissues for clinical applications.

While the majority of studies focus on bone and cartilage regeneration, recent work has shown progress in the bioprinting of other musculoskeletal tissues, such as muscles and tendons. An Integrated Tissue-Organ Printer (ITOP) was used for the fabrication of skeletal muscle structures. The approach was based on the printing of well-defined PCL patterns for directional alignment of the muscle cells, as shown in **Figure 7**. At the same time, cells were deposited using a mixture of hydrogels (gelatine, hyaluronic acid and fibrinogen), which were loaded with mouse myoblasts cells. Results showed good cell viability, and alignment along the PCL pillars/ patterns and muscle-like structures were observed after 7 days. When the constructs were implanted in vivo, they integrated with the common peroneal nerve (CPN) after 2 weeks and the muscle was seen to respond to electrical stimuli [20]. Even though bioprinting examples in this area are limited, this study provides good evidence that 3D printing can be used for the development of various fibrous tissues (muscle, tendon and ligament) where cellular alignment is a key requirement [21].
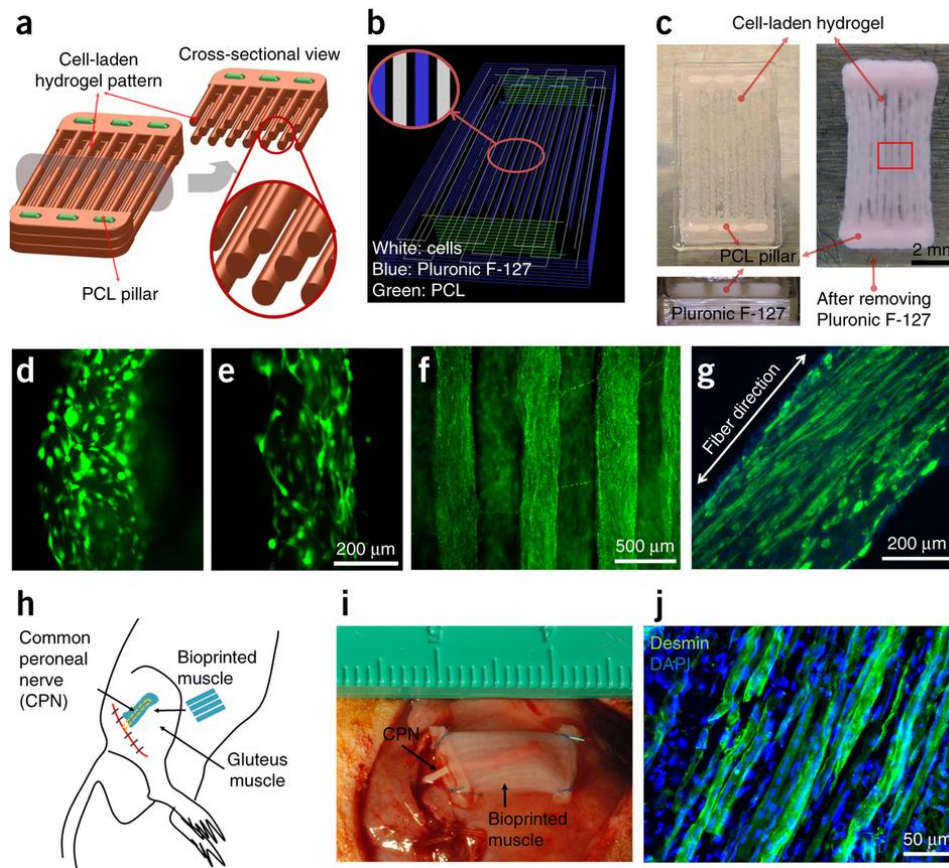
Figure 7. Bioprinting of skeletal muscle and implantation *in vivo*. (A) and (B) scaffold design; (C) scaffold fabrication; cell alignment with PCL (D) and without PCL (E); F) Live/ dead assay: green cells are alive and red cells are dead; (G) Immunofluorescent staining for myosin heavy chain of the 3D printed muscle after 7D differentiation. The encapsulated myoblasts aligned along the longitudinal direction of the fibre structure; (H) schematic of the ectopic implanted scaffold in vivo; (I) implanted scaffold next to the common peroneal nerve (CPN) and (J) immunostaining for Desmin. Adapted from [20] with permission from Nature publishing group.

While various materials have been used as bioinks for printing cell-free and cell-laden constructs, cells alone in the form of tissue spheroids have also been investigated for 3D bioprinting. Printed cells have a fluid nature and over time, they fuse together to form more complex cell aggregates that can potentially lead to tissue formation [22]. In the literature, tissue spheroids have already been successfully used for cartilage tissue engineering [23-25]. However, successful production of constructs using tissue spheroids is still in its infancy and focus needs to be applied on utilising 3D printing technologies to help with scale-up, reproducibility and formation of more complex structures [26,27].

Breakdown of the materials used as bioinks for bone and cartilage bioprinting in the last 15 years, show some interesting trends. The majority of all the cell-free approaches used materials such as PCL, β-TCP or hydroxyapatite **(Figure 3)**. In contrast, alginate was the most popular material for cell-laden bioinks (25%) and this was followed by PCL (21%) and collagen (9%). Alginate was applied due to its good printability, while PCL is a biocompatible mechanical strength enhancer of the cell-laden hydrogels. Notably, there is greater variety in the materials used for cell-laden printing than cell-free printing.

3D printing has been successfully applied in a variety of ways to address the growing demand for more robust musculoskeletal therapies. Nevertheless, the use of the technology for medical purposes is still in its infancy. There is need for further research and development in both 3D printing technology and bio-ink formulations for successful translation of this technology in future.

Biomaterials	Cells	Printing Technique	Application	Construct Morphology	Mechanical Properties	Advantages (A)/ Disadvantages (D)	Reference
PLGA + PLA (cartilage) and PLGA + tricalcium phosphate (TCP) (bone) with gradients at interface	Ovine Articular Chondrocytes	Inkjet: TheriForm™	Osteochondral	3 regions: 4 mm cloverleaf bone region with 55% porosity, 1.2 mm transition region with three gradient sections 2 mm cartilaginous region with 90% porosity and staggered 250 µm channels.	Tensile data: a. tensile strength 1.6-5.7 MPa b. elastic modulus 83-233 MPa Compressive data: a. yield strength 2.5-13.7 b. elastic modulus 54-450 MPa Diameter shrinkage: cartilage region 8.3% and adjacent transition zones 3.8%	A: Homogeneous cell seeding (material gradients at the interface) and no delamination. D: Compressive properties of the bone region of the construct are lower than those of cancellous bone.	[28]
Hydroxyapatite (HA)	Mouse Pre-Osteoblasts (MC3T3-E1)	Indirect writing (powder + binding solution)	Cartilage	Internal structure: walls that all stand in 45º to the x-axis with 1.2 mm of distance between them with 500 µm interconnecting channels.	Shrinkage after sintering: 18-20% in all directions	A: Cells cultured in static and dynamic conditions. Multiple cell layers on the surface of the HA granules (static) and cell proliferation inside granule cavities (dynamic). D: Mechanical properties not evaluated.	[29]
PLGA	Human Foetal Osteoblasts	Inkjet: ZPrinter 310 PLUS™	Bone	6 mm in diameter and 6 mm in height with interconnected channels. 1 mm pores and 55% porous. Rough Macro porous surface.	Compressive strength: 7.8 ± 3.1 MPa Compressive Young's modulus: 77.2 ± 10.8 MPa	A: Mechanical properties mimic human cancellous bone and supports osteoblasts proliferation. D: Mechanical properties still lower than the ones of human cortical bone.	[30]
PCL/ HA (shifted pattern)	Human Osteosarcoma (MG 63)	Bioplotter	Bone	$5 \times 5 \times 5$ mm³ scaffolds. Square lattice with 380-400 µm strands to generate porous structure. 600 µm pores with 92.55% porosity and. Shifted patterns.	Compressive modulus: ~22 MPa	A: Promotes cell attachment, proliferation and differentiation. Increased cell attachment by shifted pattern structure. D: Low compressive modulus.	[31]
Mesoporous bioactive glass (MBG) + Alginate	Human Bone Marrow-derived MSCs (hBMSC)	Bioplotter	Bone	$8 \times 8 \times 8$ mm³ square lattice scaffolds. 50% to 67% porosity. Internal structure: micro- and macro-pores with Nano channels (5 nm).	Compressive strength: 0.4-1.6 MPa Compressive modulus: 1.4-6 MPa Shrinkage after drying at room temperature: ~30%	A: Good mechanical properties with improved cell attachment compared to pure alginate only. Promotes cell proliferation and differentiation. D: Mechanical properties decrease after incubation with simulated body fluid.	[18]
Collagen + Alginate + Silica	Mouse Pre-Osteoblasts (MC3T3-E1)	Low temperature Bioplotter	Bone	Multi-layered cylindrical struts (324-389 µm) with mesh-like interconnected structure. Highly porous (>78%) with 468–481 µm average pore size.	Tensile Young's modulus: 1.96 ± 0.19 MPa Max. tensile strength: 0.12 ± 0.03 MPa Compressive Young's modulus: ~0.2-0.3 MPa	A: Biocompatibility, osteo-induction and production of bone-like HA. Silica improved mechanical properties compared to collagen + alginate hydrogels only. D: 2-step scaffold fabrication and cell seeding, with >7-day coating process that can cause blocked pores.	[11]
Silicon-doped Nano Crystalline HA + PCL + Carbon Nanotubes (CNT)	Human Osteosarcoma (MG 63)	Pneumatic EnvisionTEC 3D Bioplotter®	Bone	Multi-layered lattice. 7 layers with 6 mm diameter and 3 mm height. Interconnected square 450-700 µm pores.	Compressive strength: ~4 MPa Compressive elastic modulus: 50 MPa	A: CNTs improve cell attachment. 2% CNT scaffolds improve mechanical properties and electrical conductivity. D: Scaffolds loaded with more than 2% CNTs decrease compressive resistance and porosity (40 %).	[9]
GelMA	MG63 osteoblast-like cells Primary normal human	Customised bioprinter	Bone	Pores size 400 µm, thickness 750 µm Hydrogel with 8% GelMA	Before Crosslinking: storage modulus 100 Pa After UV Crosslinking: storage modulus 1000 Pa	A: Storage modulus permits printing of the hydrogel before crosslinking, and UV-crosslinking ensure suitable mechanical properties to stimulate osteoblasts proliferation. GelMA hydrogel has successfully been used to coat titanium. D: Low cell viability	[32]

	osteoblasts (NHOst)					
PCL + PLGA + Duck Beak	New Zealand White rabbit *in vivo* study with 5 mm critical defects	Bioprinting: multi head pneumatic syringe dispenser	Bone	$3 \times 3 \times 20$ mm oblong scaffolds with 77.3% porosity and 2.787 μm pores.	Compressive strength: 17 MPa	**A:** Promotes repair and de novo bone formation. High compressive strength compared to PCL/ PLGA implanted scaffolds. **D:** Irregular scaffold shape and pore structure/ distribution.	[33]
PLA+HA	*In vivo* implantation of MSCs from New Zealand Rabbits	Desktop 3D printer (Dot Go 3D Technology Corporation, Xiangtan, China)	Anterior Cruciate Ligament	Screw-like scaffold, 10 x 2.1 x 2.1 mm Pores ~290 μm	N/A	**A:** *In vivo* work showed good bone/ graft interface and successful tendon healing within bone tunnel. **D:** No mechanical test and scaffold not representative of physiological environment.	[34]

Table 1. Cell-free approach for bioprinting of musculoskeletal tissues.

Biomaterials	Cells	Printing Technique	Application	Construct Morphology	Material Properties	Advantages/ disadvantages	Reference
Lutrol F127 + Matrigel® + Alginate + Methylcellulose + Agarose	Goat Bone Marrow Stromal Cells	Pneumatic EnvisionTEC 3D Bioplotter®	Bone	20x20 mm rectangular 3D scaffolds with 300 µm spacing between fibres and 150 µm layer thickness.	N/A	**A:** Cell viability not affected by printer nozzle. Materials promoted cell proliferation and differentiation into osteogenic lineage. **D:** Hydrogels stiffness leading to fusion of adjacent layers, and no transverse pores.	[35]
Alginate	Human Articular Chondrocytes + Human Mesenchymal Stem Cells (hMSCs)	BioScaffolder™ pneumatic system	Osteochondral	Ten-layer rectangular 3D scaffolds (10×10 mm) with spacing between fibres of 0.8-2.5 mm. 100 µm layer thickness.	Viscosity: 0.5-1000 Pa. s	**A:** Controlled cell distribution/ encapsulation in hydrogels. Combine multiple cell types. **D**: Poor mechanical strength of alginate and fused transversal pores.	[10]
Cell-laden Alginate surrounding cell-free PCL (cell-free)	Human Chondrocytes (C20A4)	BioScaffolder™ pneumatic system	Hard tissue	6×60×2 mm rectangular lattice scaffolds with 2 µm fibre spacing.	Young's modulus: ~6.5 MPa	**A:** PCL improves mechanical properties of alginate. **D:** High deposition temperatures of PCL are detrimental to cell viability in alginate.	[36]
PCL + PLGA + Atelocollagen	Mouse Pre-Osteoblasts (MC3T3-E1)	Bioprinting: multi head pneumatic syringe dispenser	Heterogeneous Tissue	Hybrid scaffold with alternated layers of synthetic and natural materials, with 400 µm fibres.	N/A	**A:** Scaffold promotes cell proliferation and good viability. **D:** No consideration of mechanical properties.	[37]
PCL + Hyaluronic acid + Atelocollagen	Human Mesenchymal Stromal in Rabbit Knee	Bioprinting: multi head pneumatic syringe dispenser	Osteochondral	Hydrogels with 250 µm and 500 µm pores, between 250 µm PCL fibres. 5 mm rabbit knee defect filled.	N/A	**A:** Multilayered constructs without chemical or physical crosslinking. PCL allowed cell rich hydrogels with controlled structure. **D:** No consideration of mechanical properties.	[38]
Poly (ethylene glycol) dimethacrylate (PEGDMA)	Human Articular Chondrocytes	Thermal Inkjet Printer: Hewlett-Packard Deskjet 500	Cartilage	Cylindrical osteochondral plugs, 4 mm in diameter and 2 mm in depth.	Compressive modulus: 321.06 ± 43.99 kPa Swelling ratio: 6.10-11.80%	**A:** Simultaneous photo-polymerisation during 3D printing to maintain precise cell position during layer-by-layer assembly. Integrated layers decrease delamination risk. Compressive modulus comparable to native human articular cartilage. Biocompatibility and promotion of chondrocyte growth. **D**: Compressive module lower than non-printed PEGDMA due to thermal degradation.	[15]
Gelatine-fibrin Matrix	hMSCs + hUVECs) + Human Neonatal Dermal Fibroblasts	Aerotech AGB 10000 pneumatic syringe dispenser	Bone	Hydrogel in a 3D perfusion chip (725×650×125 mm).	Viscosity: ≤1000 Pa. s Shear elastic modulus: 1-10000 Pa Plateau modulus: 300-5000 Pa	**A:** Proliferation and differentiation into osteogenic lineage around vessels perfused with osteogenic medium. Cells survive for more than 6 weeks. Thick (>1 cm) vascularised construct. **D:** Weak mechanical properties of the construct due to gelatine and fibrin properties (not assessed).	[12]

					Yield stress: 700-9000 Pa		
Alginate + PCL	Porcine MSCs	3D Discovery multi-head pneumatic bioprinting system	Bone	Human vertebrae-like structures with orthogonal, 1 mm PCL fibres in cell-laden alginate hydrogel.	Compressive modulus: 1600 ± 100 Pa	**A:** PCL reinforced mechanical properties of alginate. Construct promoted endochondral ossification and vascularisation post implantation. **D:** Co-printing of MSC-laden alginate with PCL not possible in smaller diameter constructs (<6 mm). Alginate and PCL not crosslinked, which could be a problem under high mechanical loads. Transition of the cartilage matrix into bone conducted *in vitro* and not *in vivo*.	[13]
Hydroxyapatite + Alginate + PVA	Mouse calvaria 3T3-E1 cells	HyRel System 30M with modified EMO-25 extruder	Bone	7-layer porous cylinders with 1.5 cm diameter and 0.2 cm height.	Storage Modulus: 275-3572 Pa	**A:** PVA-HA improve printability and viability. Good mechanical properties and scaffold integrity after 14 days. **D:** Simple structures printed and did not test cell differentiation. Invasive cell viability testing (rupture of the scaffold and incubation with sodium citrate), which may have effected remaining cells within scaffold.	[39]
Alginate + Collagen	Pre-osteoblasts (MC3T3-E1)	DTR2–2210T, Dongbu Robot, Bucheon, South Korea with a dispenser and an aerosol humidifier (Tess-7400; Paju, South Korea)	Bone	Porous structures 15 x 15 x 3.6 mm	Storage modulus: 5-500 Pa Loss modulus: 1-200 Pa Viscosity: 5-200 Pa. s	**A**: Presence of ECM components gives suitable microenvironment. Good cell viability and proliferation. **D**: Poor mechanical properties and not all the tested gels permit cell proliferation and maturation to osteoblasts.	[40]
Nano-fibrillated cellulose/ Alginate	Human nasal chondrocytes and human hBMSCs	INKREDIBLE printer (CELLINK)	Cartilage	15 x 15 x 3 mm	Compressive stress: 14.9 kPa at day 0 and up to 88.2 kPa after 2 months post-implantation	**A**: In vivo implantation in nude mice for 60 days. **D**: Size of samples for compressive tests too small.	[41]
Nano-fibrillated cellulose + Alginate + Hyaluronic acid	Induced Pluripotent Stem Cells (IPSCs) + chondrocytes	3D Discovery (RegenHu)	Cartilage	7 x 7 x 1.2 mm	N/A	**A**: Co-culture permitted iPSCs differentiation into chondrocytes. Obtained hyaline cartilage-like tissue. **D**: Absence of mechanical testing.	[19]

Gelatine/ Hyaluronic acid/ Fibrinogen + PCL	Mouse C2C12 myoblasts 3T3 Fibroblasts Scaffolds implanted into nude rats	Integrated Tissue-Organ Printer (ITOP)	Skeletal muscle type II	15 x 5 x 1 mm	Compound muscle action potential: 3.6 mV	**A:** Good cell viability and induced nerve integration. **D:** Muscle function lower than positive control and did not investigate the therapeutic efficacy.	[20]
Porcine tibialis anterior muscle decellularised ECM + PCL	Mouse C2C12 myoblasts	Integrated composite tissue/organ building system (ICBS)	Skeletal muscle	Parallel, diamond and chain architectures	Viscosity: shear thinning from 50 to 0.1 Pa.s Ultimate tensile stress: 2-3.5 KPa Elastic modulus: 9-12 KPa	**A:** Good mechanical properties, structure and architecture compared to collagen hydrogels widely used for tissue regeneration. The bioink provided a suitable microenvironment for the cells. **D**: No in vivo assessment.	[42]
Hyaluronic acid/ Fibrinogen/ Gelatine + Polyurethane (PU) or PCL	C2C12 myoblasts (with PU) and 3T3 fibroblasts (with PCL)	Integrated Organ Printer	Muscle-tendon unit	Cross sections 20 x 5 x 1 mm 10% overlap region	Young's modulus: 45 MPa for PCL part Ultimate stress strain: 4.5-5.5 MPa for 3 PCL, interface and PU part	**A:** The scaffold was elastic in the muscle half and stiff on the tendon side. **D:** Mechanical testing of the whole scaffold is missing.	[43]

Table 2. Cell-laden approach for bioprinting of musculoskeletal tissues.

3. Musculoskeletal bioinks and their characteristics

Bioinks are an integral part of the bioprinting process. Most frequently, they are defined as hydrogel materials used for the encapsulation of cells in 3D bioprinting [44]. However, this definition is very limited and there are a number of examples where biological materials without cells are also termed as bioinks [45]. Opinion on the exact definition of bioink remains divided. From the literature it is clear that cell-only [46] , cells with supporting materials (both synthetic and natural hydrogels) [13] and biomolecules without cells (BMP2) [47] are also referred to as bioinks.

In the musculoskeletal context, the scenario is even more complex. As seen from the literature review in the previous section, musculoskeletal tissues have been bioprinted using three approaches: cell-free, cell-laden and combination of both approaches (i.e. multimode printing of synthetic polymers, along with encapsulated cells). The definition of bioinks becomes even hazier as the commonly used definition of encapsulated cells within material becomes very limited in its scope and application. We anticipate that as bioprinting research advances through the development of both hardware (3D printers) and novel materials which support this process, the need for an accurate and more inclusive definition will become apparent. Here we will focus on the various bioink used for musculoskeletal applications as reported in literature and define the requirements for the fabrication of functional MSK tissues. Figure 8 shows physical-chemical, biological and fabrication requirements for musculoskeletal bioinks. Most of these requirements are similar to soft tissue bioprinting, however they become specific for musculoskeletal applications when additional mechanical stiffness and rigidity is required to fabricate structurally competent tissues.
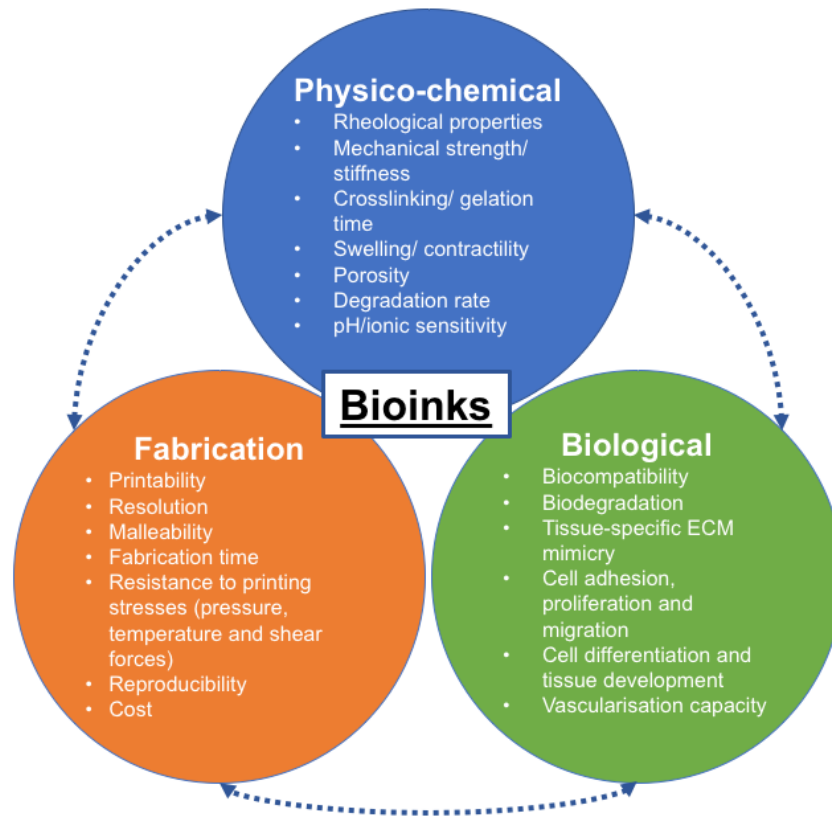
Figure 8. Characteristic requirements for bioinks in musculoskeletal tissue fabrication.

In the body, cells are found in highly organised environments, which are rich in water, nutrients and growth factors [48]**. Due to their significant water content, hydrogels have been identified as a primary material for bioprinting. Additionally, their hydrophilic nature allows hydrogels to retain large volumes of water without preventing a variety of crosslinking methods to be applied during fabrication of 3D networks [49]. In addition to this, hydrogels can be formulated to respond to various external stimuli such as temperature, electric or magnetic fields, light, pressure and sound vibrations before, during or after printing process [50]. Chemical factors including pH, solvent composition, ionic strength and molecular species also affect hydrogel properties. Therefore a good understanding of these parameters on printability, stability in both in vitro and in vivo environments become essential.

Materials for bioprinting must be biocompatible and mimic natural cellular or tissue environment [51,52]. Specifically, materials used for cell encapsulation must mimic the natural environment of cells and it has been demonstrated that hydrogels based on extracellular matrix components permit this [53,54].

In terms of fabrication, printing materials should exhibit good printability and sufficient mechanical properties for cellular support and maintenance of the 3D structures [51]. For example, since highly viscous hydrogels are prone to clogging phenomena in the nozzles of extrusion-based printers, shear-thinning of some hydrogels, such as hyaluronic acid and peptide gels, can be advantageous [55,56]. However, it is important to adapt these hydrogels so they are able to 'self-heal' and maintain their printed structure once deposited [57]. Gelation time, along with the capacity to respond to physiological shear, tensile and compressive stresses, are other key parameters in bioprinting, which determine whether a printed construct can maintain its structure in a physiological environment [58,59].

Hydrogels for bioprinting of musculoskeletal tissues can be classified as natural or synthetic depending on their origin [60], [61] and the most common ones used in the literature are summarised in *Table 3*.

Most natural hydrogels are based on components of the mammalian ECM, even though polymers from alternative sources such as algae are gaining interest [62]. Natural hydrogels show significant bioactivity compared to synthetic materials due to the intrinsic presence of biomolecules used for signalling, adhesion, biocompatibility and self-remodelling [2]. While bioactive components are important for cell growth and differentiation, the application of natural materials can lead to batch-to-batch variability, immunogenic reactions and disease transmission [63]**. Interestingly, it has been observed that natural polymers such as hyaluronic acid, laminin, fibronectin and collagen are more susceptible to cell-driven biodegradation [64].

It is important to note that as well as individual components of the ECM, decellularised ECM has similarly been successfully utilised as bioinks in the printing of tissues analogues [65,66]. Tissue decellularised ECM can be obtained using chemical, physical, and biological treatments and provides an excellent representation of the natural ECM environment [67]. At the same time, decellularised ECM can lead to non-homogeneous cell seeding and immune reactions, if cellular components are not fully removed [67]. Furthermore, decellularisation treatments can damage the natural ECM and show poor mechanical properties in the material.

Synthetic materials are advantageous in terms of reproducibility and ease of processing compared to natural polymers. These polymers can sometimes lead to immunogenic reactions after partial degradation but this can be controlled and accelerated with the addition of matrix metalloproteinases, which show excellent biocompatibility [68,69]. Furthermore, synthetic materials can be tailored to form complexes with ECM proteins by covalent crosslinking.

These covalently-bound molecules can be adhesion proteins or growth factors that enhance cell response within the hydrogels [58].

Material	Description	Tissue	Reference
Natural hydrogels			
Alginate	Polysaccharide derived from seaweed, which can be ionically crosslinked with $CaCl_2$. A: Fast gelation, general ease of use and low cost. D: Low swelling properties can limit cell survival and growth in the long term, and weak mechanical properties (Compressive modulus ~10 KPa). $CaCl_2$ crosslinker can be cytotoxic at high concentrations.	Bone	[13,70]
Chitosan	Polysaccharide derived from chitin and most commonly obtained from the exoskeletons of crustaceans. Commonly crosslinked using genipin or glutaraldehyde. A: Good biocompatibility, biodegradability, anti-inflammatory/ antibacterial properties, and good printability. Structure similar to GAGs in cartilage. D: Slow gelation and weak mechanical properties (Young's modulus in compressive mode for non-crosslinked and genipin crosslinked films is 38.7 KPa and 87.3 KPa). Glutaraldehyde crosslinking is cytotoxic.	Bone and Cartilage	[71]
Collagen	Protein composed of glycine, praline and arginine to form tropocollagen fibres of diameters ranging from 50 to 200 nm. A: Good swelling properties and biocompatibility. D: Weak mechanical strength (mean peak stress: 0.76 MPa), poor printability and expensive.	Bone and Cartilage	[72]
Fibrin	Fibrin is a non-globular protein present in the blood produced during blood clotting. Can be enzymatically crosslinked. A: Good biocompatibility, swelling and gelation properties. Good printability. D: Weak mechanical properties and expensive.	Bone	[73]
Gelatine	Protein obtained from hydrolysed collagen, which can be crosslinked using temperature and enzymes. A: Low cost, biocompatible, biodegradable with high-cell adhesion. D: Poor printability. Often found coupled to methyl acrylate (MA) to form GelMA (crosslinked with UV light and harmful to cells), which has significantly increased mechanical properties and improved printability compared to gelatine.	Bone and Cartilage	[74,75]
Hyaluronic acid (HA)	Polysaccharide and major component of ECM. (Photo) chemical crosslinking. A: Good biocompatibility, good swelling ratio (0-45) fast degradation rates (100 to 0% residual mass in 8 days). D: weak mechanical properties (storage modulus 400-1000 Pa, loss modulus 3-30 Pa) and limited printability due to shear-thinning.	Bone	[76]
dECM	Tissue decellularized ECM can be obtained using chemical, physical, and biological treatments. A: Representative of natural ECM environment, tissue-specific, guides for stem cell differentiation and good biocompatibility. D: Non-homogeneous cell seeding and immune reactions if cellular components remain. Decellularization treatments can damage natural ECM. Poor mechanical properties: max storage modulus of 300 Pa and 0-20 Pa loss modulus.	Bone	[77]
		Skeletal muscle	[42]
Synthetic hydrogels			
Poly ethylene glycol (PEG)	Synthetic polymer. Commonly crosslinked via chain-growth and step-growth polymerisation, but can also be crosslinked using radiation and other chemical/ physical methods. A: FDA approved and does not trigger immunological responses. The material is soluble in water and organic solvents, and has low protein adhesion properties. Good diffusion of	Bone	[17]

	nutrients and oxygen, and cell migration. When coupled to methyl acrylate, it shows high swelling properties (swelling ratio from 37.88-100.93%) and good biocompatibility. Compressive modulus of 30-65 KPa and 1.63-6.99 cP viscosity. Good printability. D: Often lack bioactive molecules.		
Self-assembling peptides	Self-assembling peptide-based gels. A: Versatile properties that can be easily tailored by adjusting chemicals and physical parameters. Good extracellular matrix mimicry, while biocompatible and biodegradable. Bioactive molecules can be incorporated. D: Peptide gels are degraded by cellular proteolytic enzymes to cause shrinkage (20% of hydrogel volume in 12 days). Consequently, the mechanical properties become weaker (decrease from 50 to 10 Pa in 12 days).	Bone	[78]

Table 3. Summary of the most popular hydrogels used for musculoskeletal bioprinting. Materials are categorised as natural or synthetic hydrogels, and the advantages (A) and disadvantages (D) of each material are described.

4. Challenges in bioprinting of musculoskeletal tissues

In addition to the hardware used for bioprinting, material availability and their selection are significant challenges and limitations for the success of bioprinting in musculoskeletal tissue fabrication. Materials/ bioinks composed of naturally derived materials are limited in their application due to batch to batch variability and often lack the mechanical strength required to mimic the *in vivo* environment of native musculoskeletal tissues [59]. In the literature, this issue is often resolved by combining the natural inks with stronger biocompatible materials, such as PCL, PLA and PLGA [28,33] as described previously.

Furthermore, bioinks are presently limited by their printability and resolution. Materials are often required to be viscous in order to maintain the morphology of printed structures and improve mechanical strength, but this can lead to blockages and unreliable material deposition. Importantly, this has adverse effects on the print quality and resolution, which can be detrimental to achieving the highly hierarchical structures in the tissues of the musculoskeletal system.

Tuneable, synthetic bioinks can provide a wide range of desirable properties, including controlled mechanics, degradation and printability. However, the techniques required to synthesise and crosslink these materials can cause cytotoxicity and prevent the ability to incorporate cells during the print process. For example, the monomers and photo initiators in some printable materials are toxic, but following UV crosslinking, the polymers formed are biocompatible and can support cell survival [79,80].

Further, printing of mechanically stable gelatine in the form of GelMA traditionally requires UV light during the print process and can damage cells. Recently, this challenge has been overcome by developing GelMA that can be crosslinked using visible light [81]. Crosslinking of natural bioinks can similarly be associated with cytotoxicity but this can often be alleviated by using low crosslinker concentrations or replacing with non-toxic analogues. For example, calcium chloride is used for crosslinking alginate, whereas glutaraldehyde is used for crosslinking collagen, gelatine, or chitosan. Calcium chloride is not toxic at low concentrations, while glutaraldehyde can be replaced with alternatives like genipin, during the crosslinking of collagen, gelatine and chitosan [82-84].

Hydrogels are the most utilised materials in bioprinting, due to their high-water content and parallels to native ECM. However, these biomaterials show poor mechanical properties so compromises must be made when considering characteristics such as composition, printability and mechanical strength. In the bioprinting of musculoskeletal tissues this has been overcome to an extent by incorporating multiple printing technologies at the same time. This approach permits deposition of materials with good mechanical properties and cell-laden bioinks, within a single engineered construct [13,36].

Furthermore, the fundamental layer-by-layer nature of most printing techniques leads to difficulties in producing complex and hollow structures. This can be resolved by incorporating sacrificial materials for structural support during the fabrication process but this also increases the technological complexity, cost and time of the printing. Once the resolution, reproducibility, speed and customisation of current printing technologies have been defined and optimised, bioprinting can provide cost-effective and high-throughput systems for drug screening and tissue replacement. Importantly, robust methods for construct maturation and long-term maintenance, as well as quality control measures for bioprinted tissues, need to be considered in parallel with the technological advances of printers. Regulatory concerns, such as the ethics of stem cells and the use of Class II medical devices are also key factors to contemplate, for the success of bioprinting approach. Furthermore, at present there are no specific 3D bioprinting regulations defined by the Medicines and Healthcare Products Regulatory Agency (MHRA) in the UK or Food and Drug Administration (FDA) in the USA.

5. Summary and future perspective

Printing of natural ECM-based materials embedded with cells provides significant advantages for tissue engineering, including biocompatibility, robust control of cell distribution and density within the scaffold. Nevertheless, traditionally materials have been produced and seeded with cells afterwards, and this can also be applied to printed constructs. In fact, this approach permits scaffolds with enhanced mechanical properties and resolution that natural materials often lack for musculoskeletal applications. Furthermore, co-printing of cell-laden and cell-free materials has been revealed to be beneficial for musculoskeletal tissue engineering applications [13,31,36]. All these approaches (cell-laden, cell-free and a combination of both) aim to obtain a complex, living tissue able to develop and mature, using the same fundamental technology.

The bioprinting literature suggests that the definition of bioinks remains unclear, considering various components such as cells, biomolecules, synthetic materials either alone or in combination, are defined as bioinks. Consensus in this matter is required. With the development of new bioinks in the future, nano-biofabrication of organs will become a reality and this will help to alleviate the increasing organ shortages worldwide. More specifically, technological advances in material science and engineering will permit versatility, nano-scale resolution and controllable distributing of cells and biomaterials, for a range of biomedical applications, including musculoskeletal repair.

The literature shows that bioprinting of muscles, tendons and ligaments is still a challenge, however promising progress has been made in bioprinting of bone and cartilage. Stiff materials have been combined with natural cell-laden hydrogels to form composite constructs that are mechanically stable with the ability to mimic the native ECM environment of osteochondral tissues. Further, it has been demonstrated that these scaffolds can be combined with stem cells to permit osteochondral development in vivo [13]. Progress has even been made in the bioprinting of vascularised bone [14]. Bio-sensors for bone formation [85], and protein and DNA arrays of stem cells [86,87] have already been bioprinted, while next generation printable materials for controlling osteochondral cellular microenvironments are also in development. Nevertheless, these examples are all proof of concept studies and significant validation and development of next generation bioinks and their printing process is required in the future.

Fundamentally, the success of bioprinting in tissue engineering is heavily reliant on improvements in bioink properties, printing technologies, vascularisation of tissues, and controlled scaffold and cell maturation. Crucially for bone and cartilage applications it is

important to improve the mechanical properties of bioinks and to maximise the resolution of the printed constructs. Innovative bioinks with benefits for musculoskeletal applications that are currently being developed include, dynamic switchable hydrogels with local variations in the density and size of collagen fibres throughout 3D tissues [88,89], and oxygen releasing biomaterials [90,91].

In conclusion, bioprinting promises to be an important tool to fabricate complex tissue and organs. However, there are significant challenges to be resolved in terms of technological advances. Research to date has laid strong foundations and promise for the feasibility in manufacturing artificial organs, including musculoskeletal tissues. Bioprinting and the use of bioinks remain developing and expanding multidisciplinary fields of research with substantial potential for the future successes of tissue engineering and regenerative medicine.

Executive Summary

Introduction

- Successful translation of tissue engineering and regenerative medicine research relies on efficient, robust and cost-effective fabrication techniques.
- Bioprinting is an automated additive manufacturing process that permits the fabrication of 3D structures by selectively depositing biological materials layer-by-layer.

Bioprinting and its role in musculoskeletal tissue fabrication

- Bioprinting in the literature shows promise for musculoskeletal regeneration.
- Greater understanding of the native environment in tissues and organs, is required to maintain cell viability.
- Printer hardware needs to be developed to combine cell health with desired biomaterials characteristics.

Musculoskeletal bioinks and their characteristics

- Bioinks can be printable biological materials that must be compatible with the biological, chemical and physical requirements of native tissues.
- To be suitable for musculoskeletal applications, a bioink must have suitable mechanical strength for cellular support and maintenance in 3D structures.
- A range of natural and synthetic materials have been used for musculoskeletal applications.
- Natural materials provide the biological properties for tissue development but lack the mechanical strength of bone and cartilage. Synthetic materials provide a solution to this challenge but are less bioactive.
- The most promising examples in the literature, combine cell-free and cell-laden printing.

Challenges in bioprinting of musculoskeletal tissues
• Bioinks cannot currently mimic the environments that cells experience *in vivo*.
• Printing living cells is challenging because there are multiple variables that need to be controlled and optimised when cells are combined with biomaterials.
• Hard tissues require mechanical strength that natural hydrogels often lack and synthetic materials can be detrimental to cell viability.

Summary and future perspective

- Bioinks must meet the following requirements: biocompatibility, biodegradability, good printability and sufficient mechanical strength for cellular support and maintenance of the 3D structures.
- The use of bioinks in bioprinting remains a developing and expanding multidisciplinary technology with substantial potential for the future successes of tissue engineering and regenerative medicine.

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