RegMedNet Spotlight

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Beyond cells: innovations in tissue engineering

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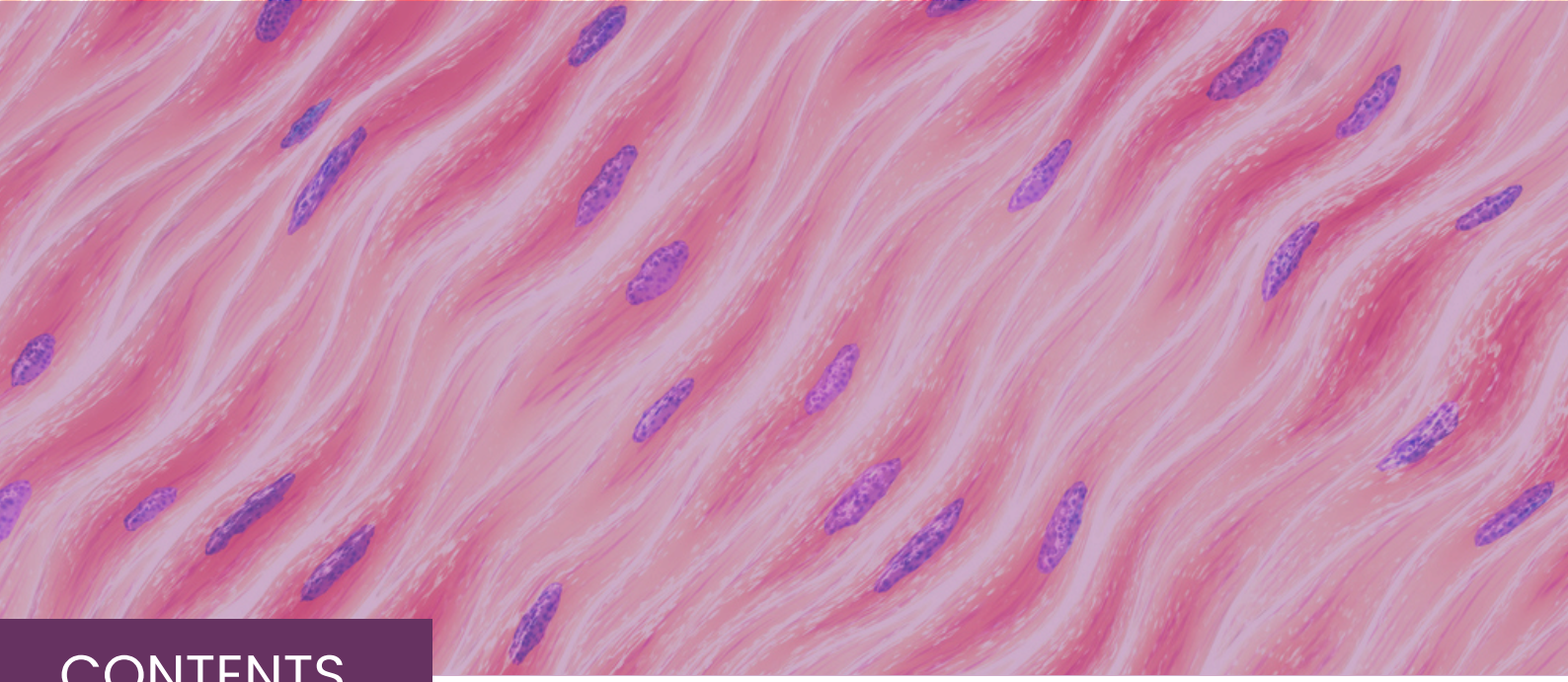

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FOREWORD

We are pleased to present you with this eBook to conclude our **Spotlight feature** on tissue engineering.

Tissue engineering in regenerative medicine is a dynamic, multidisciplinary field that involves the application of principles from engineering, biology and materials science. Promisingly, tissue engineering offers versatile solutions for treating a variety of conditions, including injuries, degenerative diseases and congenital defects, by restoring damaged or lost tissues.

This eBook will delve further into the applications of tissue engineering for regenerative medicine and investigate innovative strategies for improving tissue engineering approaches, such as utilizing smart scaffolds and *in situ* 3D bioprinting.

We hope you enjoy reading these expert insights with us.

Megan Giboney
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Tissue engineering: exploring innovations, approaches and future frontiers

As Professor of Tissue Engineering at Newcastle University, Che Connon has successfully combined his passion for entrepreneurial activities with notable academic achievements.

A bio-physicist by training, Professor Connon has a real passion for applied biology. Che and his team have been putting their minds to solving complex issues in cell and tissue engineering and bioprocessing, often by taking radical approaches. This has frequently meant not simply taking a bigger hammer to a particular problem but trying to see it from a different angle. Often this involves an improvement in the fundamental understanding of cell or tissue biology, notably using the cornea as an exemplar.

Che Connon
Professor of Tissue Engineering
Newcastle University

1 What excites you the most about the field of tissue engineering?

In regenerative medicine, the prospect of applying tissue engineering to repairing or replacing lost or damaged tissues holds a lot of promise. For example, if someone is unfortunate enough to lose a foot or a hand, or in the case of suddenly failing organs.

Another application that excites me is industrial tissue engineering, which involves taking all of the things we've learned about creating tissues for medical applications and applying them to other areas, such as cultured meat, new advanced materials or leather replacements.

And something that certainly excites me about tissue engineering is what I call super tissue engineering. So, having tissues that act beyond their normal capabilities and exceed current biological limitations.

For example, take the cornea – it's the main refractive element of the eye and it protects the eye from damage. But imagine if you engineered a cornea so it did all those things, but it also had other capabilities. So maybe it changed color or had telescopic capabilities or had a camera embedded in it. Similarly, envisioning other parts of the body where a hand could have beyond normal human strength, for example.

While some of these concepts are in very early stages or speculative, others, like cultured meat and lab-grown leather, are much more advanced.

2 How do combinatorial approaches improve tissue engineering and what role do they play in your research?

We take multiple approaches to create these engineered tissues, but ultimately my lab is taking more of an engineering biology approach, focusing on bioinspired cues. We're particularly interested in the physical cues that cells receive in the body and using them to guide cellular growth. We start with the cells and build up from there, rather than using more of a top-down approach, which starts with the scaffold and then integrates the cells. I prefer the bottom-up approach because it acknowledges the complexity of tissue functionality.

Tissue engineering: exploring innovations, approaches and future frontiers

I think an interesting example has been curvature in cell culture. For 50, maybe 100 years, people have been growing cells on flat surfaces. But there are no flat surfaces inside a body. So, we started growing corneal stromal cells on very gently curved surfaces, similar to the curvature you see in the cornea. The perceived wisdom was that there was no way cells could sense that very low angle of curvature.

However, when we grew cells on a curved glass surface, the cells could sense that difference and organized themselves in a particular way. We found that if we put them around the edge of a glass dome they would form a circular pattern around the very edge and then some of the cells would turn 90 degrees and climb in straight lines, forming a radial pattern that followed the curvature of the dome.

In the cornea, we know that aligned cells produce aligned collagen and aligned collagen forms a more transparent tissue than non-aligned collagen. So, there's a really interesting relationship between the physical environment you put cells in and the function of the tissue that's formed.

We have also explored how mechanical cues, such as the softness or stiffness of the cellular environment, impact cellular behavior, particularly corneal epithelial stem in the stem cell niche. We found that by manipulating these cues and combining them with curvature, we can drive cell differentiation and guide tissue formation to create more functional tissues.

3 What is your perspective on the role of bioprinting in tissue engineering?

I think bioprinting was initially seen as an exciting technology. When we think back to around 10 years ago, 3D-printed ears were used to demonstrate the applicability of 3D printing in medicine because an ear was immediately recognizable.

But I always wondered, where are the hordes of the earless? Why do we need to print ears? In addition, I think the problem with 3D bioprinting is that, while it was relatively easy to print something that looks like an ear, it lacked the function of an ear.

Ultimately, the functionality of a tissue comes down to the organization of the microscopic components, particularly the arrangement and hierarchical structures of proteins. The resolution of those components can be in the tens of nanometers, whereas the resolution of bioprinting is in the hundreds of micrometers.

Some suggest maturing 3D printed tissues to allow the cells to organize themselves but that takes several weeks, negating the main advantage of 3D bioprinting being a rapid way of doing things.

We recognized that 3D printing probably wouldn't solve critical issues in tissue engineering so returned to a bottom-up approach, getting the cells to do the work. And if the cells do the work then you don't really need them to be 3D printed to begin with.

4 How do you see the field of tissue engineering evolving in the next decade, and what impact do you think it will have on regenerative medicine?

One trend I think we will see is the continued increase in the use of tissue engineering for non-medical purposes. This is where I think we're seeing the biggest advances in the field in terms of the industrial scale-up of tissue engineering that is required to deliver on the promise of alternative proteins with cultivated meat or lab-grown leather. I

foresee that some of the progress made in this space will find use for itself in regenerative medicine in the future and tissues for regenerative medicine will be able to be produced using very high-throughput, scalable methods further reducing the cost and complexity of receiving those tissues.

The other area that I think is very exciting is what I call super tissue engineering, which I mentioned earlier. Essentially super tissue engineering is engineering tissues that go beyond normal functionality and it's something we're working on at Newcastle University. An example I've already mentioned is adding additional capabilities to the cornea, for example, by embedding an electronic chip. This could be employed to address clinical needs, such as monitoring intraocular pressure, which is linked to various eye diseases.

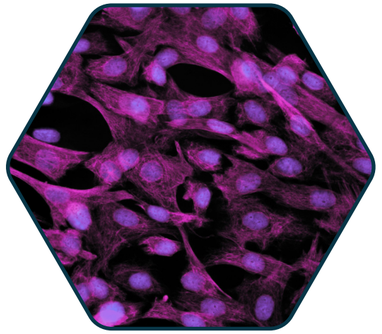
One of our PhD students is designing a chip that can sense changes in the curvature of the cornea and relay that information to give real-time measurements of intraocular pressure.

I expect to see an increase in concepts like this that combine microelectronics with tissue engineering, rather than inserting microelectronics directly into the body. This approach could leverage the biocompatibility of engineered tissues to mitigate rejection issues and help to enhance the functionality of microelectronics.

This interview is part of the RegMedNet Spotlight feature on tissues engineering.
Discover expert opinions on this topic by visiting our feature homepage.

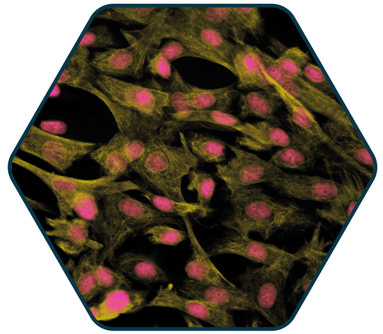
View the feature >>>

Engineered Tissue Scaffold and Cell Therapy Considerations for Patients with Volumetric Muscle Loss and Other Large Muscle Injuries



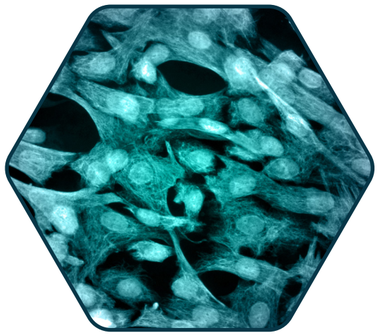
Scaffold Characteristics

- Degradable over time as new muscle tissue forms
- Specific mechanical properties: high tensile strength & flexibility
- Potentially stiffer than surrounding tissue to shield healing area



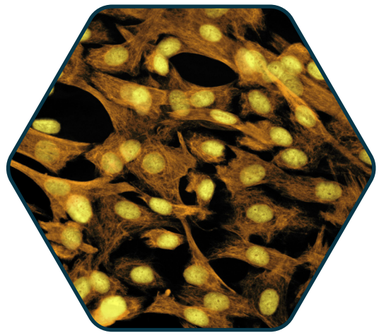
Cell Survival After Implantation

- Promote vascularization with factors like VEGF, nutrients, and oxygen
- Induce innervation to prevent muscle atrophy
- Provide signals and growth factors to mimic native tissue environment



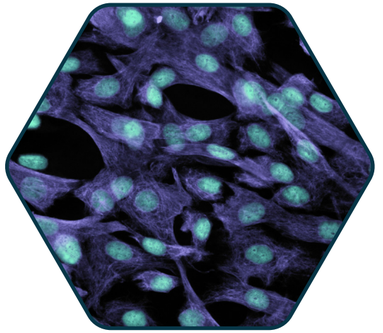
Cell Attachment to Scaffolds

- Cell adhesion via binding sites (e.g. RGD sequences)
- Mechanical properties and surface characteristics impact cell behavior
- Channels/alignment can guide formation of myotubes
- Cells remodel scaffold as it degrades and tissue reforms



Cell Selection for Muscle Regeneration

- Isolate young, healthy satellite cells and fibroblast/ adipocyte progenitors
- Prevent premature differentiation and senescence during expansion
- Recruit additional native progenitor cells from surrounding tissue



Scaffold Attachment Considerations

- Use sutures or adhesives to connect scaffold to native tissue
- Provide a bridge for cell migration and signaling
- Balance immobilization and gradual mechanical loading
- Pre-vascularization strategies for larger scaffolds

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Review

Regenerative Medicine

Engineered cells along with smart scaffolds: critical factors for improving tissue engineering approaches

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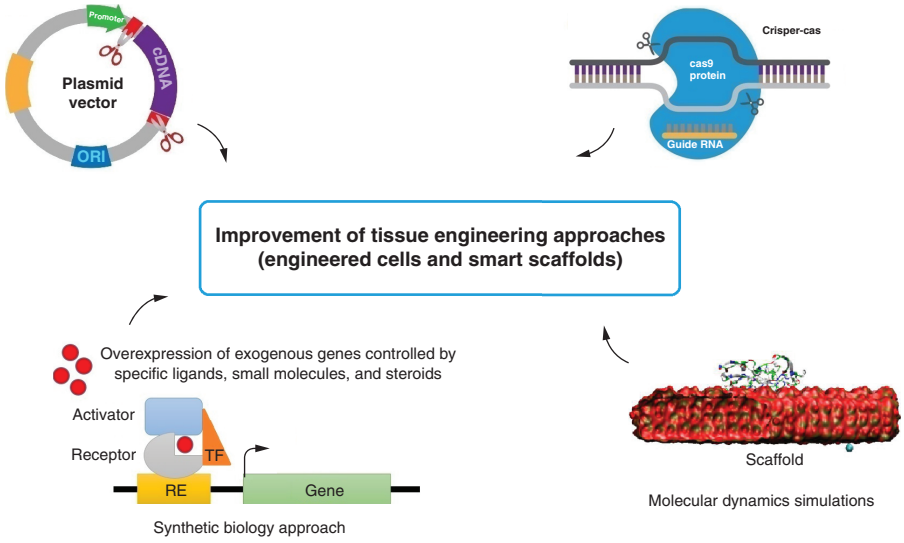
In this review, gene delivery and its applications are discussed in tissue engineering (TE); also, new techniques such as the CRISPR-Cas9 system, synthetics biology and molecular dynamics simulation to improve the efficiency of the scaffolds have been studied. CRISPR-Cas9 is expected to make significant advances in TE in the future. The fundamentals of synthetic biology have developed powerful and flexible methods for programming cells via artificial genetic circuits. The combination of regenerative medicine and artificial biology allows the engineering of cells and organisms for use in TE, biomaterials, bioprocessing and scaffold development. The dynamics of protein adsorption at the scaffold surface at the atomic level can provide valuable guidelines for the future design of TE scaffolds /implants.

Tweetable abstract: Engineered cells and smart scaffolds for improving tissue engineering approaches.

Graphical abstract:



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Keywords: CRISPR • engineered cells • gene therapy • molecular dynamics • MSC • smart scaffold • synthetic biology • tissue engineering

Future Medicine

Tissue engineering (TE) is a multidisciplinary field of study; its main aim is reconstructing damaged tissues. In addition, it plays an essential role in drug development and disease modeling [1,2]. TE attempts to regenerate damaged tissues by combining body cells with extremely porous scaffolds that function as tissue regeneration templates for creating new tissue. The scaffolds made from a range of biomaterials and various fabrication processes have been employed to regenerate multiple tissues in the body. When fabricating a scaffold for tissue engineering, several essential factors are necessary, regardless of tissue type, including mechanical qualities, biocompatibility, scaffold architecture, biodegradability and manufacturing technique [3].

The physical and biological features of scaffolds can influence cell fate [4]. Gene/cell-based treatments are the most advanced regenerative medicine category, including over 500 clinical studies in phase II and III [5]. A potential strategy for TE is offered by scaffold-based gene therapy through transfecting cells to improve the sustained expression of the interest protein or by silencing target genes related to numerous illnesses. One interesting technique is the delivery of genes via TE scaffolds [6,7]. The biological activity of the cells might be regulated to achieve the desired result by transfecting certain sequences of genes into cells, silencing or overexpressing the original gene. Gene therapy when combined with TE scaffolds, allows a more precise, regulated and prolonged release of therapeutic agents than traditional methods of directly administering growth factors [8]. Generally, two methods have been presented to introduce the genes into a cell including nonviral vectors and viral vectors (Tables 1–3) [9]. There are several advantages of gene delivery including, sustained local delivery, regulated gene expression, less expensive, suitable for intracellular gene products and the possibility of treatment only once; however, like other treatment approaches, gene therapy may have limitations such as safety concerns about viral vectors, failure in many clinical studies and low pDNA transfer efficiency in the case of nonviral vectors [10].

The clustered regularly interspaced short palindromic repeats-associated protein9 system (CRISPR-Cas9) is a natural bacterial defensive mechanism to fight phage infection. Using this approach in humans, plants and animals, it is possible to precisely manipulate almost any particular genome sequence using a guide RNA short stretch, allowing the correction of mutations that cause disease, elaboration of the gene function associated with disease development and progression and activating deactivated cancer suppressor genes using an effector domain and a fusion protein of nuclease-deficient Cas9 [59–61]. The current applications of CRISPR-Cas systems in TE have highlighted tissue architecture construction, disease model development, cell differentiation and immune response circumvention. Stem cells are excellent cell sources with enormous promise for TE applications [62].

Synthetic biology employs biological insights and engineering concepts to develop and construct novel biological functions and sophisticated artificial systems, which do not exist in nature. Promoter and operator sequences, termination sites, ribosome binding sites, transcription factors and reporter proteins are the molecular biology processes employed as key components in synthetic biology [63]. The TE, scaffold and material development, stem cells, bioprocessing control and gene therapy are the fields where synthetic biology can significantly advance in these areas in the future [64]. Biosynthesis and regulated release of therapeutic molecules, scaffold material synthesis, stem cell regulation and programming cells to arrange themselves into novel tissues are all examples of synthetic biological approaches used in the area of TE [65].

Molecular dynamics (MD) simulation is a valuable modeling technique for investigating atomic-level interactions between biomaterials and biomolecules [66]. Some experimental assays investigate the attachment and proliferation of cells to the TE scaffolds. However, they might not be simple, especially for large specimens, since the fabrication technique and the biocompatibility test need to be performed for each of the proposed constructs, which can be expensive and require a lot of time. Therefore, a computational approach that estimates the adhesion of cells to the constructs may be effective for reducing the number of specimens and accelerating the process of optimization. This approach is beneficial for composite constructs, which are made up of several polymers and for developing new polymers [67].

In this review, we discuss the various studies performed based on genetic engineering or gene delivery in the field of TE. We will also look at novel applications of the scaffold from the perspective of new techniques in genetic engineering, such as the CRISPR-Cas9 system, synthetic biology and MD simulation. It is suggested that by using new approaches, researches in the field of TE is out of the traditional mode and takes on an intelligent form. In general, stem cells can be a good source for genetic engineering purposes in TE. Also, with the synthetic biology strategy, controllable tissues can be designed. With the CRISPR approach, cell differentiation or immune pathways can be genetically engineered or scaffolds with a high binding tendency can be designed with computational

Table 1. Summary of scaffold-mediated gene delivery for tissue engineering in bone.

Tissue	Types of vector	Types of cells	Scaffold mediated gene delivery	Active molecules (genes)	Study model/animal model	Application	Ref.
Bone	Nonviral vector – DNA nanoparticles	MSCs	Collagen sponges reinforced by incorporating of PGA fibers	*BMP-2* and PEI-Ac80 solutions	*In vitro* and *in vivo*	3D scaffolds-encapsulated DNA nanoparticles increase osteogenic differentiation of mesenchymal stem cells	[11]
Bone	Ultrasound-mediated gene delivery	MSCs	Collagen	*BMP-6*	*In vivo*	effectively treat nonhealing bone fractures in large animals (mini-pigs)	[12]
Bone	Adenovirus	bMSCs	Permineralized silk scaffolds	*BMP-2*	Rat	A permineralized silk scaffold could be used to build tissue-engineered bone for mandibular bony defects	[13]
Bone	Lentivirus	ADSCs	β-TCP scaffold	*BMP2* and *BMP7*	Rat	ADSCs co-transfected with *BMP2* and *BMP7* on β-TCP scaffold can have therapeutic potential for bone defects	[14]
Bone	Adenovirus transfer vector	bMSCs	Hydroxyapatite scaffolds	*OPG*	Rat	The OPG-BMSC-HA constructs can orchestrate osteoporotic-related bone defect reconstruction	[15]
Bone	Adenovirus	MSCs	The combination of nano-calcium sulfate/platelet-rich plasma gel scaffold	*BMP2*	Rat	In critical-sized bone defects, scaffolds containing *BMP2*-modified MSCs successfully promote bone regeneration	[16]
Bone	Adenovirus	bMSCs	Porous ceramic scaffold (OsteoBone™)	*BMP2*	Rabbit	OsteoBone scaffold could be an ideal carrier for gene-enhanced bone tissue engineering	[17]
Bone	Ad5–hVEGF165-EGFP or mock vector	Endothelial progenitor cells	Nanohydroxyapatite/collagen/poly(L-lactic acid) scaffolds	*hVEGF-165*	Rat	The hVEGF165/endothelial progenitor cells-nanohydroxyapatite/collagen/poly(L-lactic acid) composites may have potential application in the repair of segmental bone defects	[18]
Bone	Lentiviral vector	bMSCs	CPC scaffolds	*BMP-2*	Rat	Calvarial repair and bone regeneration can be achieved successfully using lentiviral-mediated *BMP-2* gene therapy in conjunction with CPC scaffolds	[19]
Bone	Nonviral delivery	MSCs	Bioprinted MSC alginate scaffolds	*BMP-2*	Mice	The results proposed a model for combining 3D printing and non-viral gene therapy	[20]
Bone	pIRES2-EGFP plasmid	bMSCs	Calcium alginate gel	*hIGF-1*	*In vitro*	Mosaicplasty with *hIGF-1* gene-enhanced tissue engineering can improve the outcome of large osteochondral lesions in the weight-bearing region	[21]
Bone	Lentiviral vector	MSCs	CDM scaffold	*IL-1Ra*, *BMP2*, *TGF-β3*	*In vitro*	The findings suggest the possibility of patient-specific tuning of both growth factor secretion and scaffold remodeling	[22]
Bone	Nonviral vector – chitosan nanoparticles	MSCs	Collagen hydroxyapatite scaffold	*VEGF* and *BMP-2*	Rat	This naturally derived cell-free gene-activated scaffold can accelerate bone repair in critical-sized bone lesions	[23]

Ad5: Adenovirus Type 5; ADSC: Adipose-derived stem cell; BMP: Bone morphogenetic protein; bMSC: Bone marrow-derived stem/stromal cell; β-TCP: β-tricalcium phosphate; CDM: Cartilage-derived matrix; CPC: Calcium phosphate cement; IL-1Ra: IL-1 receptor antagonist; MSC: Mesenchymal stem cell; OPG: Osteoprotegerin; PGA: Poly(glycolic acid); PEI: Polyethylenimine; PLGA: Poly lactic-co-glycolic acid.

Table 1. Summary of scaffold-mediated gene delivery for tissue engineering in bone (cont.).

Tissue	Types of vector	Types of cells	Scaffold mediated gene delivery	Active molecules (genes)	Study model/animal model	Application	Ref.
Bone	Nonviral vector – chitosan nanoparticles	MSCs	Collagen hydroxyapatite scaffold	*BMP-2* and *BMP-7*	Rat	Optimizing the plasmid construct improves the functionality of gene-activated scaffolds and enhances bone formation in critical-sized defects	[24]
Bone	Nonviral vector – chitosan nanoparticles	–	Chitosan-based thermosensitive hydrogel scaffold	*BMP-2*	Rat and beagle dog	The finding suggest that this platform is a potential candidate as a targeted, multi-effect scaffold for the endogenous repair of alveolar bone	[25]
Bone	Nonviral vector – pMC A2-minicircle backbone vector	Skull-derived osteoblasts	PLGA scaffold	*BMP-2*	Mouse	This approach is an efficient, long-term non-viral gene delivery system that may be used to enhance tissue repair	[26]
Bone	pEGFP-N1 vector	–	Collagen and gelatin hydrogels	*BMP-2*	Mouse	According to the findings, gelatin hydrogel was more effective than atelocollagen as a substrate for local gene delivery and may be a better material for inducing bone regeneration	[27]
Bone	Nonviral vector –nanohydroxyapatite	MSCs	Alginate hydrogels	*TGF-β3* and *BMP-2*	–	The results indicate that the developed gene-activated alginate hydrogels were capable of transfecting encapsulated MSCs and directing their phenotype toward chondrogenic or osteogenic differentiation, depending on whether *TGF-β3* and *BMP2* were delivered together or separately	[28]
Bone	Nonviral vector – PEI complexes	hPLFs and hGFs	Collagen	*PDGF*	Rat	In this model, the non-viral gene-delivery system demonstrated to prolong the inflammatory response, thereby inhibiting alveolar bone regeneration *in vivo*	[29]
Bone	Nonviral vector –calcium phosphate conjugated PEI	hMSCs	Collagen-nanohydroxyapatite	*TGF-β3* and *BMP-2*	*In vitro*	This improved delivery system may facilitate the healing process for osteochondral tissue regeneration by enhancing the functionalized composite graft	[30]

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strategies. It is hoped that these new research approaches will lead to further advances in TE and regeneration medicine and be able to help cure humans faster.

Cells engineering strategies in TE

Gene-modified cells

Genetic engineering can be used to induce the production of various proteins and soluble factors with diverse uses, like cytokines, chemokines, growth factors, transcription factors, mRNAs and enzymes [68]. Nonviral vectors (like polymers or lipids) and viral vectors (including retroviruses, lentiviruses, adenoviruses and adeno-associated viruses) have been employed to overexpress genes in cells like mesenchymal stem cells (MSCs) [69]. In the presence of the appropriate growth factors, MSCs could experience chondrogenic differentiation, making them a suitable cell source for cartilage TE [70]. To investigate the impact of the expression of exogenous *Chm-1* in MSCs, Chen

Table 2. Summary of scaffold-mediated gene delivery for tissue engineering in cartilage.

Tissue	Types of vector	Types of cells	Scaffold-mediated gene delivery	Active molecules (genes)	Study model/animal model	Application	Ref.
Cartilage	Lentiviral vectors	hMSCs	3D woven poly(e-caprolactone) scaffold	*TGF-β3*	*In situ*	Leads to chondrogenic lineage differentiation and the accumulation of cartilage-like ECM	[31]
Cartilage	pcDNA3-TGF-β1	bMSCs	Chitosan	*TGF- β1*	Rabbit	The results indicate that *TGF β1* gene-modified cartilage tissue engineering using MSCs, chitosan, and liposomal transfection can improve cartilage repair	[32]
Cartilage	Adenovirus	bMSCs	CS/SF scaffolds	*CNP*	Rat	The CS/SF scaffolds containing of *CNP* gene-modified BMSCs demonstrate promising approaches for repairing focal cartilage defects	[33]
Cartilage	Lipofectamine/pDNA	MSCs	Poly(lactide-co-glycolide)/fibrin gel construct	*TGF-β1*	Rabbit	The PLGA/fibrin gel hybrids were applied as a 3D platform and results showed that the full-thickness defects of cartilages were repaired using neo-tissues with smooth surfaces and similar histology characteristics to the normal cartilage	[34]
Cartilage	Adenoviral vectors	bMSCs	DBM scaffold	*BMP-2* and *TGF-β3*	Pig	The scaffold is biocompatible and may induce cartilage regeneration to repair joint cartilage defects. This technology has the potential to be used effectively to repair cartilage lesions *in vivo*	[35]
Cartilage	Adenovirus	bMSCs	PGA scaffold	*SOX9*	Rabbit	The findings showed the essential role of *SOX9* in repairing cartilage lesions *in vivo* and demonstrated that SOX9 had the potential and advantage for tissue engineering applications	[36]
Cartilage	Nonviral vector –poly(ethylene oxide)-b-poly(l-lysine);	rbMSCs	PLGA scaffold	*TGF-β1*	Rabbit	The study showed that the synthesized construct has low cytotoxicity and high biocompatibility, making it an effective candidate for cartilage regeneration *in vivo*	[37]
Cartilage	Lipofectamine	hMSCs	Alginate scaffold	MicroRNA-221	*In vivo*	The findings suggest that silencing miR-221 has a pro-chondrogenic role *in vivo*, which opens up new opportunities for using hMSCs in cartilage tissue engineering	[38]
Cartilage	Lentiviral vector	ADSCs	3D woven poly(e-caprolactone) (PCL) fibers	*IL-1Ra*	*In vitro*	Researchers used advanced textile manufacturing and scaffold-mediated gene delivery to create large anatomically shaped cartilage constructs with controlled anti-cytokine therapy delivery	[39]

ADSC: Adipose-derived stem cell; ASC: Adipose-derived stem cell; BMA: Bone marrow aspirate; BMP: Bone morphogenetic protein; CNP: C-type natriuretic peptide; CS/SF scaffold: Chitosan/silk fibroin scaffold; DBM: Demineralized bone matrix; ECM: Extracellular matrix; IL-1Ra: IL-1 receptor antagonist; MSC: Mesenchymal stem cell; PLGA: Poly lactic-co-glycolic acid; rAAV: Recombinant adeno-associated virus; SOX9: SRY-box transcription factor 9.

Table 2. Summary of scaffold-mediated gene delivery for tissue engineering in cartilage (cont.).

Tissue	Types of vector	Types of cells	Scaffold-mediated gene delivery	Active molecules (genes)	Study model/animal model	Application	Ref.
Cartilage	Adeno-associated virus (rAAV-5)	Chondrocytes	Fibrin carrier without cells	*IGF-1*	*Ex vivo*	The results showed that *IGF-I* gene therapy could stimulate cartilage healing and improve the mechanical properties of repaired grafts	[40]
Cartilage	Lentiviral vector	Chondrocytes	PLGA scaffold	*Integrin β1*	*In vitro*	The results hypothesized that the periodic mechanical stress and *integrin β1* up-regulation in chondrocytes could improve the quality of tissue-engineered cartilage	[41]
Cartilage	rAAV	hMSCs	Fibrin-polyurethane scaffolds	*SOX9*	*In vitro*	This system represents a promising approach for implanting in focal cartilage defects and improving cartilage repair	[42]
Cartilage	rAAV	hBMA	3D-woven poly(e-caprolactone; PCL) scaffolds	*SOX9*	–	The results indicate the therapeutic potential of the rAAV-modified marrow concentrates within 3D-woven PCL scaffolds for repair of focal cartilage defects	[43]
Cartilage	Baculoviral vector	rASCs	PLGA scaffold	*TGF-β3* and *BMP-6*	*In vitro* and *In vivo*	The Baculoviral vector-engineered rASCs that persistently express *TGF-β3*/*BMP-6* improved chondrogenesis, *in vitro* cartilage construct production, and *in vivo* hyaline cartilage regeneration	[44]
Cartilage	rAAV	Chondrocytes	Poly (ethylene oxide) (PEO) and poly (propylene oxide) (PPO) (poloxamer and poloxamine) polymeric micelles	*TGF-β1*	*In vitro*	Delivery of therapeutic rAAV vectors by PEO-PPO-PEO micelles could be potential tools to remodel human osteoarthritis cartilage	[45]

ADSC: Adipose-derived stem cell; ASC: Adipose-derived stem cell; BMA: Bone marrow aspirate; BMP: Bone morphogenetic protein; CNP: C-type natriuretic peptide; CS/SF scaffold: Chitosan/silk fibroin scaffold; DBM: Demineralized bone matrix; ECM: Extracellular matrix; IL-1Ra: IL-1 receptor antagonist; MSC: Mesenchymal stem cell; PLGA: Poly lactic-co-glycolic acid; rAAV: Recombinant adeno-associated virus; SOX9: SRY-box transcription factor 9.

et al. transfected the MSCs of rabbits with adenovirus-containing chondromodulin-1 gene (Ad5-Chm-1) and then these cells were seeded inside a natural coral TE scaffold; vascularization was reported to be inhibited by *Chm-1* and maintained chondrocyte phenotype *in vivo* in Ad5-Chm-1-transfected MSCs. According to the results, *Chm-1*-modified MSCs can be an optimized source of the cell for cartilage TE [71]. In a study, an adenovirus-incorporated gene was used to seed *VEGF* gene-modified endothelial progenitor cells onto scaffolds of bladder acellular matrix grafts to improve blood supply in tissue-engineered bladders in a porcine model. The findings suggested that using endothelial progenitor cells in conjunction with *VEGF* gene therapy might be a viable strategy for improving blood supply in bladder TE. The most effective angiogenic growth factor, *VEGF*, was purposely selected for experiments since vascularization seems to be a vital element in TE, particularly in large and critical-size bone lesions [72,73].

The possibility and benefits of establishing a novel approach for bone TE were investigated by Jiang and *et al.* via rat bone marrow mesenchymal stem cells (BMMSCs) and β-tricalcium phosphate, lentivirus vector modified with human *VEGF 165* gene (*hVEGF165*) and human bone morphogenetic protein 2 gene (*hBMP2*). According to the findings, this approach utilizing β-tricalcium phosphate in combination with lentiviral-transduced MSCs co-transfected with *VEGF165* and *BMP2* was beneficial; therefore, offering a unique treatment approach for bone deficiencies [74]. Dai and colleagues conducted a study on bone TE. They performed their study using the human cytotoxic T lymphocyte-associated antigen 4-Ig (*CTLA4-Ig*) gene, which is a popular ligand for CD80/CD86. The findings showed that hBMMSCs with the *CTLA4-Ig* gene could develop properly to osteoblasts *in vitro*,

Table 3. Summary of scaffold-mediated gene delivery for tissue engineering in skin and other tissues.

Tissue	Types of vector	Types of cells	Scaffold-mediated gene delivery	Active molecules (genes)	Study model/animal model	Application	Ref.
Skin	PEI	hUVECs	Poly(DL-lactide)–poly(ethylene glycol) (PELA) scaffold	*VEGF* and *bFGF*	Rat	DNA condensation and multiple delivery strategies could help fully vascularize engineered tissues and effectively regenerate blood vessel substitutes	[46]
Skin	Nonviral vector – N,N,N-trimethyl chitosan chloride (TMC)	hUVECs	Collagen–chitosan	*VEGF-165*	*In vitro*	Full-thickness burn healing using a plasmid DNA encoding *VEGF-165* activated collagen-chitosan scaffold	[47]
Skin	Nonviral – N,N,N-trimethyl chitosan chloride (TMC)	Porcine/fibroblasts	Collagen–chitosan/silicone membrane	siRNA *TGF-* $\beta 1$	*In vitro*	Inhibition of scarring and full-thickness skin regeneration using the RNAi functionalized collagen-chitosan/silicone membrane	[48]
Skin	Lipofectamine™ 2000	HaCaT cells	Collagen gels	*hEGF*	Rat	HaCaT cells that have been genetically modified with the *hEGF* gene may be promising seed cells for developing genetically modified skin substitutes that can effectively secrete *hEGF* to accelerate wound repair and regeneration	[49]
Skin	2-dimethylaminoethyl Methacrylate/2-propyl acrylic acid (DMAEMA/PAA)	Human cervical cancer cells	PUR scaffold	siRNA *GAPDH* gene	Diabetic rats	This platform contains pH-responsive siRNA-loaded nanoparticles that incorporate into a biodegradable polyurethane (PUR) scaffold and is a promising approach for local gene silencing in nonhealing skin wounds	[50]
Skin	Pegylated poly-L-lysine (PLL-g-PEG) polymer	COS-7 cells	Fibrin hydrogels	*HIF-1*α	Healthy or diabetic rats	In cutaneous wounds, the PLL-g-PEG-dependent delivery of an oxygen-insensitive variant of *HIF-1* successfully stimulates differential gene expression and induces an initial angiogenic response	[51]
Nerve	Lipoplexes	HEK293T	ECM coated PLG 3D bridges	Firefly luciferase and β -galactosidase	Rat	The study examined lipoplex deposition onto PLG scaffolds and translated surface-mediated DNA delivery techniques for *in vivo* transgene expression in the spinal cord	[52]
Nerve	Lipoplexes	Primary dorsal root ganglion (DRG) neurons and HEK293T cells	Poly(lactide-co-glycolide) (PLG) bridges	*NGF*	Rat	To pattern DNA within a 3D polymer scaffold, surface-mediated DNA delivery was combined with multiple channel spinal cord bridges	[53]

BDNF: Brain-derived neurotrophic factor; ECM: Extracellular matrix; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HaCaT cells: Human epidermal keratinocyte cells; HUVEC: Human umbilical vein endothelial cell: HIF-1α: Hypoxia-inducible factor 1-alpha; HEK293T: Human embryonic kidney 293 cells; hUMSC: Human umbilical cord mesenchymal stem cell; hESdC: Human embryonic stem cell-derived cell; MSC: Mesenchymal stem cell; PGA: Poly(glycolic acid); PLGA: Poly lactic-co-glycolic acid; PLLA: Poly-l-lactic acid; PEI: Polyethylenimine; PUR: Polyurethane; SDF-1: Stromal cell-derived factor 1.

Table 3. Summary of scaffold-mediated gene delivery for tissue engineering in skin and other tissues (cont.).

Tissue	Types of vector	Types of cells	Scaffold-mediated gene delivery	Active molecules (genes)	Study model/animal model	Application	Ref.
Nerve	Liposomal vector ScreenFect^{R}	MSCs	An adhesive peptide-modified hydrogel scaffold	*BDNF*	*In vitro*	The research demonstrated successful delivery of BDNF to the lesion site in the spinal cord. The delivery strategy had a significant effect on the repair of nerve tissue	[54]
Adipose	Adenovirus	hUMSCs	Silk fibroin 3D scaffolds	*insulin*	Rat	The adenovirus-transfected hUMSCs are compatible with the silk fibroin scaffold, and adenoviral transfection of the human *insulin* gene can be applied to construct tissue-engineered adipose tissue	[55]
Urethral	Retroviral pMSCV – VEGF165-GFP vector	Bladder urothelial cells	Decellularized rabbit artery as scaffold	*VEGF165*	Rabbit	The findings suggest that *VEGF* gene therapy may be an appropriate strategy to promote blood supply in tissue engineering for the treatment of urethral damage or loss	[56]
Skeletal muscle	Lipofectamine 2000	Myoblasts	Calf collagen scaffolds	*VEGF-165* and *SDF-1*	Rat	The combination of a collagen sponge seeded with myoblasts and gene therapy is a suitable strategy for the construction of well-vascularized skeletal muscle	[57]
Vascularizing tissue	Nonviral vector –biodegradable polymeric nanoparticles	hMSCs and hESdCs	PLGA/PLLA scaffolds	*VEGF*	*In vitro* and *In vivo*	The findings suggest that polymer nanoparticles-engineered stem cells could be used therapeutically for treating ischemic disease and vascularizing tissue constructs	[58]

BDNF: Brain-derived neurotrophic factor; ECM: Extracellular matrix; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HaCaT cells: Human epidermal keratinocyte cells; HUVEC: Human umbilical vein endothelial cell: HIF-1α: Hypoxia-inducible factor 1-alpha; HEK293T: Human embryonic kidney 293 cells; hUMSC: Human umbilical cord mesenchymal stem cell; hESdC: Human embryonic stem cell-derived cell; MSC: Mesenchymal stem cell; PGA: Poly(glycolic acid); PLGA: Poly lactic-co-glycolic acid; PLLA: Poly-l-lactic acid; PEI: Polyethylenimine; PUR: Polyurethane; SDF-1: Stromal cell-derived factor 1.

dramatically decrease immune response in a mixed lymphocyte culture model, and survive until bone tissue is created *in vivo*. As a result, *CTLA4Ig*-modified hBMMSCs may be attractive seed cells used for bone TE [75]. Deng *et al.* used adenoviral vectors and microencapsulation to create a very effective BMP-2/VEGF gene transduction and recombinant system that releases protein. BMP-2 and VEGF produced by encapsulated cells may significantly promote BMSCs to osteogenic differentiation in the co-cultures of BMSCs and encapsulated cells; furthermore, dual-gene transduction improved BMSCs osteogenic differentiation *in vitro* and enhanced bone regeneration *in vivo*. Encapsulation of genetically engineered BMSCs allowed for the adequate release of *BMP-2* and *VEGF*, effectively stimulated osteogenic differentiation of normal-cultured BMSCs and displayed bone healing of the orbital wall defect after implantation with tricalcium phosphate scaffolds *in vivo* [76]. Another research examined the ability of BMP-2 gene-transfected BMMSCs in combination with nano-hydroxyapatite/collagen/poly (L-lactic acid) (nHAC/PLA) to promote bone defect healing in rabbits. The findings demonstrated that the 3D BMP-2 transfected MSCs/nHAC/PLA construct can bone healing via genetic TE [77].

Although tissue-engineered dermis (TED) is regarded as the best therapy for skin wounds, the lack of vascular structures in these products might induce slow vascularization or transplant failure [78]. Han and colleagues investigated the medical applications of *VEGF*-expressing microencapsulated human umbilical cord mesenchymal stromal cells (hUCMSCs) in TED vascularization. A collagen-chitosan laser drilling acellular dermal matrix composite scaffold was employed in this study. To create TED, hUCMSC derived fibroblasts were cultivated

on the composite constructs. The results demonstrated that microencapsulated *VEGF* gene modified hUCMSCs might successfully increase TED vascularization and, resulting in wound healing quality [79]. Researchers have used scaffolds to transport various genes in diverse tissues, which is summarized in Tables 1, 2 & 3.

CRISPR systems & TE

Advances for gene editing like CRISPR-Cas9 in TE, bioelectronics and diagnostics are rapidly expanding [80]. Collins *et al.* recently proposed a programmable CRISPR responsive intelligent material composed of the CRISPR associated nuclease Cas12a and DNA containing hydrogels capable of delivering biological information via alterations in material characteristics. These researchers developed a platform for delivery and detection using DNA responsive hydrogels that relies less on strand displacement or structural changes in DNA crosslinkers [81].

In 2012, a new report on the manipulation of biological systems, through the use of CRISPR RNA sequences was presented for the direction of bacterial enzymes of Cas9 to identify and break DNA at specified locations [82] in order to create unique cell types which can be exploited for TE. The reprogramming of fibroblasts into neurons and skeletal myocytes has been carried out with the help of this approach. Conversion of lineage, gene manipulation and other modification approach offers the possibility of creating cell populations that can perform essential TE functions like extracellular matrix (ECM) production, degradation of the materials and interaction with the host [83]. Direct differentiation using CRISPR/Cas systems has been used in disease modeling and TE. Scientists used this system to edit human intestinal stem cells to better understand colorectal cancer (a common life-threatening malignancy in the world) in the world development. The researchers used the CRISPR/Cas approach to edit the induced pluripotent stem cells (iPSCs) genomes to create isogenic cell lines without or with mutations. These isogonic cells are useful in TE and the modelling of disease [84,85]. As a means of enhancing the osteogenic potential of adipose-derived stem cells, the CRISPR/Cas system was used to replace BMP antagonist encoding sequences (*BMP-2*) with BMP agonist-encoding sequences (noggin) [62]. The cellular mechanism that upregulates the BMP antagonist is controlled by replacing noggin with *BMP-2* in order to express the BMP agonist while noggin promotion is active. In this way, even when BMP production is inhibited, the adipose-derived stem cells population would be directed toward an osteogenic lineage. This application of CRISPR/Cas systems to direct stem cell differentiation into a specific lineage is a beneficial alternative to the frequently used clinical application of exogenous recombinant growth factors (Figure 1A). [84]

Optogenetic control for endogenous gene transcription has been achieved recently by developing a CIBN-dCas9 fusion protein that dimerizes with the transcription activator CRY2VP64 when exposed to blue light. This light-activated CRISPR-Cas9 effector enables spatiotemporal control of cell differentiation. This ability to manipulate the pattern and timing of differentiation could create tissue-engineered constructs with biomimetic tissue architecture [87–89].

Other approaches to gene delivery in TE

The goal of TE is to use cells, biomaterial scaffolds and suitable biochemical and/or physicochemical agents in other to replace or regenerate tissues or organs that have been damaged via disease or injury. Various complex biological signaling pathways are required for tissue growth and regeneration. As a result, in the field of drug and gene delivery for regenerative medicine, the co-delivery of combinations of biological factors has been evolving [90]. At the cellular and tissue level, gene delivery can improve functional recovery or regeneration of lost tissues. Nevertheless, more effective carriers are required to deliver genetic materials safely and locally [91]. Nowadays, attractive methods have been developed, such as applying 3D bioprinting containing bioinks for application in a specific cell, protein and/or gene [92]. To control the presentation of therapeutic genes to stem cells in 3D printed scaffolds, researchers have developed a hydrogel bioink that can be applied to spatially and temporally control [93]. According to these studies, MSCs were co-encapsulated with pDNA complexes encoding interested genes including osteogenic (*BMP2*) or chondrogenic (combination of *TGF-β3*, *BMP2* and *SOX9*); therefore, the pore-forming hydrogel bioinks enhanced transgene expression within the bioinks. Using the approach mentioned, it is possible to create bone-like tissues or stable cartilage. Engineering spatially complex tissues such as the osteochondral unit was made possible by using bioinks [94,95].

Using immobilized microfluidic synthesized gene-delivery nanocomplexes, researchers found that the designed methodology for *in situ* bone TE has significant potential and can easily be expanded in regenerative nanomedicine. These researchers described a controlled formation of pDNA (encoding human BMP-2) according to a scaffold of nanofibrous poly(ε-caprolactone) (PCL). These nanocomplexes were immobilized with the support of a

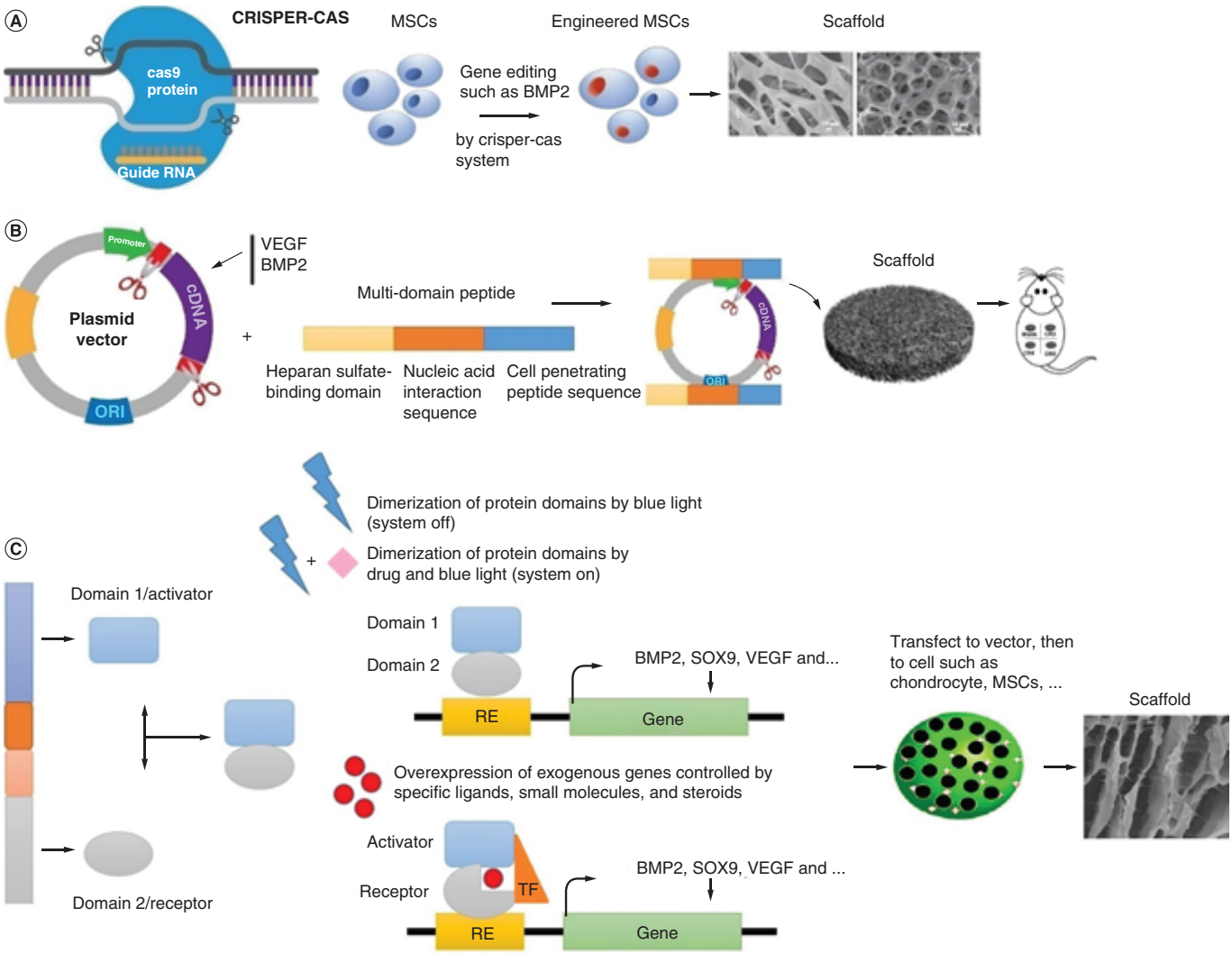

Figure 1. Different genetic engineering strategies related to tissue engineering. (A) The CRISPR-Cas system may be applied to edit genes (such as *BMP2* or other genes) to improve the differentiation potential of stem cells toward a particular lineage. **(B)** A gene-delivery system has been designed by researchers containing a GET-pDNA (a novel multidomain containing both a cell-binding sequence and cell-penetrating peptide) that enhances cell interaction and intracellular translocation of nucleic acids. This system can be inserted into the scaffold used in a cell-free model *in vitro* [86]. **(C)** Application of synthetic biology in TE and scaffolds. The optogenetic or soluble factors induction systems to control exogenous genes. Figure displays a schematic of the circuit design that enables spatial activation of synthetic circuits in distinct patterns that activates a specific gene in response to blue light along with drug; also, these systems can provide active exogenous gene expression after stimulation by soluble factors and when soluble factors are not present, the system stays off [2].
TE: Tissue engineering; TF: Transcription factors; RE: Response elements.

metalloprotease-sensitive peptide to localize the effect while also providing the environmentally sensitive presence of plasmids; in order to develop an osteogenic differentiation platform for MSCs cells, these constructs were tested on their structure, stability and functionalities [91]. Biological process development knowledge is required for sequential drug/gene delivery. The practical and translational aspects of engineering scaffold designs to acquire temporally controlled sequential delivery of genes/drugs should also be considered [90]. An interesting gene delivery system has been designed in a study by Raftery and *et al.* These researchers described a GET-pDNA (a type of pDNA with a novel multidomain that has both a cell-penetrating peptide and cell-binding sequence) that has been found to improve cell interaction and the translocation of nucleic acids inside the cell. This plasmid contains both osteogenic (*pBMP-2*) and angiogenic (*pVEGF*) genes that are inserted into scaffolds of collagen-hydroxyapatite with proven

regenerative potential for different indications. This system completely repaired critical-sized bone defects within 4 weeks (Figure 1B) [86].

Self-assembly is a process by which nanophase/molecule spontaneously forms into prepared collections. One of the interesting approaches that can be placed in the category of intelligent scaffolds is the use of 3D polyfunctional self-assembly nanoconstructs for TE. These nanoconstructs, including graphene, peptide hydrogel, fullerene and carbon nanotubes, can be used for gene delivery in TE and are promising for bone tissue regeneration. Based on 3D self-assembly nanostructures, various growth factors, including BMP-2, CCK-8, Ang-1, TGF-β1, bFGF and etc. can be delivered in cells (i.e., osteoblasts, fibroblasts, MSCs) for bone TE purposes [96]. Another work in the area of nanomedicine and TE has been done via Walsh *et al.*, these researchers designed a biocompatible, cell-free and bioresorbable scaffold containing a gene vector comprising a novel star-polypeptide biomaterial. Many types of branched polypeptide structures are called 'star-polypeptides'. Star-polypeptides are made up of linear polypeptide arms that radiate out from a central core. This study illustrated that a novel class of biomaterials in the star-polypeptides form could successfully deliver a cargo of therapeutic pDNA from a collagen-based scaffold *in vivo* with the ability to reconstruct bone tissue as a result of this delivery. The *in vitro* results exhibited that the co-delivery of *VEGF* and *BMP-2* by the 64-star-polypeptides vector can be applied to stimulate MSC-mediated osteogenesis on various collagen-based scaffolds with a diversity of macromolecular compositions [97].

Many nonviral gene-delivery systems, including lipofectamine, PEI and calcium phosphate, etc., have been used for tissue repair or engineering [98]. However, there are challenges for some of these delivery systems, including low transfection efficiency or toxicity concerns (such as PEI) that require be used of better gene delivery systems [99,100]. The application of some nonviral delivery systems, such as liposomes, has suitable properties for these purposes and forms a targeted delivery system. Using liposomes to pDNA encapsulation can reduce the availability of DNase and thus improve transfection efficiency. Accordingly, gene-delivery systems like liposomes along with scaffolds may resolve the problems of liposome aggregation and toxicity and improve long-term expression and efficiency of transfection [101]. Monteiro and *et al.* designed a gene delivery system consisting of electrospun nanofiber mesh (NFM) as a scaffold and liposomes as a gene delivery system. The pDNA containing liposomes were coated at the surface of functionalized PCL NFM. The hBMSCs cultured on *RUNX2* containing liposomes coated at the surface of electrospun polycaprolactone NFM displayed increased levels of metabolic activity and synthesis of total protein. In this study, *RUNX2* was considered for gene delivery. Because this central gene is involved in the simulation of the osteoblast phenotype, it may be beneficial for the induction of bone tissue regeneration [102]. The gene-transfer systems must be optimized to have minimal toxicity effects. The dendrimer-based delivery systems can be used for this purpose. Dendrimers are symmetrical molecules and nanoscales with a homogeneous architecture composed of tree-like branches radiating from a central core [103]. In a study, a gene-activated collagen scaffold based on polyamidoamine dendrimer (dPAMAM) was designed by Walsh and *et al.* This designed vector contained the reporter gene–pDNA (dPAMAM–pDNA polyplexes) and successfully transfected MSCs in 2D monolayer culture without causing prolonged toxicity. The results showed that the dPAMAM vector is a biocompatible and beneficial gene-delivery vector for TE application that, when combined with a type of specific composite scaffold can be used to regenerate different tissue lesions [104].

Scaffolds novel applications

Synthetic biology in TE

The advancements in synthetic biology have enabled the use of genetic circuit engineering to program cells via dynamic gene expressions, new biological functions and logic controls (AND, OR and NOT gate as control gates of the gene-expression system). Using engineered artificial mechanisms for patterning, controlled gene expression and morphogenesis is one way to produce synthetic tissues [105]. Engineered cells are the primary focus of synthetic biology; this includes generating cells that can carry out custom functions, such as synthetic biological strategies to TE [1]. The traditional tools applied to construct tissues in the laboratory do not allow for comprehensive control over the behavior of cells. Recent advances in synthetic biology have resulted in robust and flexible strategies for programming cells with artificial genetic circuits, where cell function and behavior can be controlled. Foundation for engineering mammalian cells is hierarchical, including: identifying and isolating genes of interest (such as *Runx2*, *ZNF145* and *MYOD1)*; DNA elements toolbox to design genetic circuits (promoters, transcription factors, activators/repressors, input–output circuits and controlling expression of endogenous genes); DNA transduction/transfection methods (nonviral vectors and viral vectors); selection/verification according to activation of DNA sequence FACS, PCR, Southern blotting and DNA sequencing [2].

Some approaches for modulating cellular behavior have resulted from the overexpression of individuals or combinations of genes; these researches were significant in clarifying the role of particular genes in tissue-specific phenotypes, differentiation of stem cells and improving cells' therapeutic potential [106]. Overexpression genes in the synthetic biology approach can be controlled by various factors, including soluble factor (small molecules, steroids and specific ligands) [107], optogenetic induction systems (blue light) [108], (Figure 1C) modifications of material with synergistic effects and conjugation of inducers (materials whose characteristics can be regulated in response to cell-mediated/environmental changes or particular inducers), and synthetic engineered receptors (chimeric antigen receptor [CAR] T-cell) [109]. In addition, regulation of exogenous gene expression using expression control systems such as Tet-on/Tet-off (Figure 1C) may be considered a safe way to transfer genes into cells; thus, the role of synthetic biology is clearly important here. In a study related to modifications of material with synergistic effects, Ma *et al.* designed a chondrocyte cell line with two *SOX9* and B-cell lymphoma 2 (*Bcl-2*) transgenes, which were stimulated by doxorubicin and coumermycin, respectively, to elevate functional phenotypes and cell viability of a cartilage tissue implant. Individual genes were temporally regulated in this method in order to maintain alive in an inflammatory condition and enhance chondrogenic phenotypes. The absorption of coumermycin to the gelatin fibers, led to the delivery of it in a 4 days period while doxorubicin inserted to the microspheres of PLGA delivered during 40 days. In the cells cultured on the composite scaffolds fabricated of gelatin and PLGA microparticles, the results of real-time PCR showed the dual-delivery model can induce high expression of *Bcl-2* at hours 48–72 and induce *SOX9* expression on days 8–10 [110]. For engineering skeletal muscle cells, in a technique related to the control of optogenetic based on tissue models, Sakar *et al.*, designed the artificial light-gated ChR2 (channel of rhodopsin-2) ion. Zone-specific and dynamic contractions based on the spatial or temporal presentation of light were illustrated when these cells were applied to form skeletal muscle microtissues. In addition, primary cardiomyoblasts and human embryonic kidney containing the channelrhodopsin-2 were co-cultured to generate an *in vitro* model of cardiac muscle that contracts when it is stimulated by light [111,112].

To obtain a controllable dissolution approach, a mixture of synthetic hydrogels and protein releasing, drug-inducible synthetic biological modules in cells can be employed. The researchers created an intelligent hydrogel in which increasing antibiotic concentrations caused the gel to dissolve and release a growth factor that was embedded within it in a time and dose-dependent way. These researchers believe that controlled dissolution is important for TE applications. For example, a hydrogel scaffold based on the drug-sensing gyrase subunit B (GyrB) is engineered for biopharmaceutical inducible release. GyrB was coupled to a hexahistidine tag in this system and then dimerized by the antibiotic coumermycin. When this protein was mixed with polyacrylamide that had been functionalized with Ni^{2+}-chelating nitrilotriacetic acid (NTA), it spontaneously formed a hydrogel. The addition of novobiocin inhibited GyrB dimerization and caused the hydrogel to dissolve. This dissolution might be used to cause the release of a desired protein [113–115]. Synthetic biology's major objective is to design genetic circuits that apply fine control over cell activity by endowing cells with complex capabilities such as responding and sensing. Recently, synthetic biology techniques have been used to promote stem cell differentiation into desired lineages and to engineer synthetic tissues that mediate the inflammatory environment for enhanced tissue repair [116]. It is expected that modular approaches developed in the area of synthetic biology will open new doors for the development of novel tools for tissue engineers in the future.

MD simulation in TE

MD simulation has served as an effective method for simulating various phenomena related to biotechnology at nanoscales in recent years, due to the ability to accurately model fully atomistic interactions between biomolecules and surfaces at nanoscales [117]. MD simulation has been used on a wide range of surfaces, including carbon nanoconstructs and structured surfaces and polymeric amorphous surfaces [118–120]. Protein adsorption on inorganic materials is a critical issue in a variety of fields, including biochemistry, biophysics, biomaterials and biomedicine [121,122]. It can be suggested that this process also may be important in biotechnology because performing MD simulations and a better understanding of scaffolds, the best protein selected can be achieved, and the gene of this protein can be used for the gene-delivery purposes in TE; also, with a computational approach, the adsorption of a protein can be examined with several different scaffolds to obtain the best scaffold in order to efficient adsorption of protein on their surface.

Sarmadi *et al.* applied the MD simulations to model the adsorption of proteins on heterogonous polymeric surfaces with varying percentages of PVA and PCL in a medium similar to water. For MD simulations, two major ECM proteins with similar size but different secondary structures, collagen type I and fibronectin, were

considered. Following simulation findings, the experimental analysis revealed that the more hydrophobic the surface, the greater the initial cell proliferation and cell attachment, which was especially evident in samples containing more than 70% of PCL [67]. Marquetti *et al.* studied the BMP-2's dynamic behavior on a flat gold substrate, which is commonly utilized in medical devices, using MD simulations. The adsorption of protein was studied at three various temperatures: the temperature of the room (293K), *in vivo* conditions (310K) and water boiling point (373K). Finding demonstrated that in high temperatures at the nanoscale, BMP-2 was resistant since no denaturation happened. The outcomes of this research will help understand the influence of temperature on the interaction between BMP-2 and gold substrates at the nanoscale [123]. Utesch *et al.* investigated the early stages of the BMP-2 adsorption when reaching two implant surfaces, hydrophilic titanium dioxide rutile and hydrophobic graphite, using classical MD and a hybrid model of steered molecular dynamics (SMD) combined with MD simulations. Protein surface adsorption was studied in a water environment for six various protein orientations, four side-on and two end-on. Researchers observed weak but stable adsorption on graphite. Flexible loops of the protein and hydrophobic patches were involved in the interaction with graphite, depending on the initial orientation. BMP-2, on the other hand, was only weakly adsorbing to hydrophilic titanium dioxide. Even though the protein had optimal interaction energy with the TiO_2 surface, the quick formation of a two-layer water structure prevented direct protein–TiO_2 interaction. Adsorption-induced denaturation of BMP-2 was not observed on the nanosecond time scale for either surface, hydrophilic titanium dioxide or hydrophobic graphite [124]. Oliveira *et al.* used atomistic MD simulations to study the conformation modifications of BMP-2 homodimer and monomer in water and vacuum [125]. Mücksch *et al.* applied the MD study to investigate the BMP-2 adsorption on a hydrophobic graphite surface [126]. The simulation of SMD is being developed to speed up the adsorption and desorption of proteins on material surfaces. Most researchers are merging MD and SMD simulations to study the interactions of biomaterials and biomolecules [127]. For example, Lai *et al.* used merged MD and SMD to study the osteopontin residues' mechanical behaviors on hydroxyapatite surfaces; they demonstrated that the osteopontin-hydroxyapatite interfacial features were influenced by the electrostatic attraction between calcium and acidic amino acid residues (Figure 2 [128] A).

A study with MD and simulation approaches reported that efficient and selectable delivery systems can be designed for TE purposes. According to this strategy, the cationic polymer-based nonviral vectors via two various approaches were described: preparation of PEI-based structures similar to the core and dynamic constitutional frameworks as nonviral vectors. Results exhibited that these designed systems can efficiently bind oligonucleotides of length different and even transfect genetic materials into cells. Both approaches have enormous potential for producing effective and even selective gene carriers [132].

Identifying naturally occurring binding interactions and modes between integrins and ECM elements provides a potential opportunity for computational approaches in TE. This approach can contribute to discovering unknown functional peptide motifs and developing new chemical molecules that specifically bind certain integrin. For instance, the RGD (Arg-Gly-Asp) motif because of its ability to bind various integrin types has been investigated through experimental and computational tools. As a result, the presence of an RGD motif within a specific scaffold may force conformations of the motif, which are only identified by a subset of integrin [133]. Both experimental and computational method was applied to create a small molecule antagonist (RUC-1), which is only specific to one type of integrin. The native RGD antagonist binds to various integrins and initiates a conformational switch between an open and a closed state, while the RUC-1 antagonist binds only to the αIIbβ3 integrin and does not induce a change between the open and closed state [134]. Other computational methods target membrane proteins by optimizing the peptide epitope sequence that binds the membrane receptors [135,136]. In addition, a scaffold based on computational methods with the feature of self-assembly peptide can be designed. These self-assembling peptides should contain a sequence capable of self-assembly and a sequence motif that is accessible and recognized via integrin in order to control their behavior. This is an opportunity for computational methods to design the favorable sequence for obtaining specific mechanical characteristics (such as mechanical stiffness), as well as rational optimization for accessible and active conformations of the integrin-binding motifs (Figure 2B [133]).

Advantages of new biological approaches

Gene delivery has a number of benefits, the most notable of which are prolonged localized delivery, controlled expression of genes, lower costs, appropriate for intracellular gene products and the possibility to undergo therapy just once [10]. Transduction refers to the gene transfer by viral vectors. In gene therapy, herpes simplex viruses, adeno-associated viruses, lentiviruses, adenoviruses and hybrid viruses are utilized as viral vectors. These vectors are

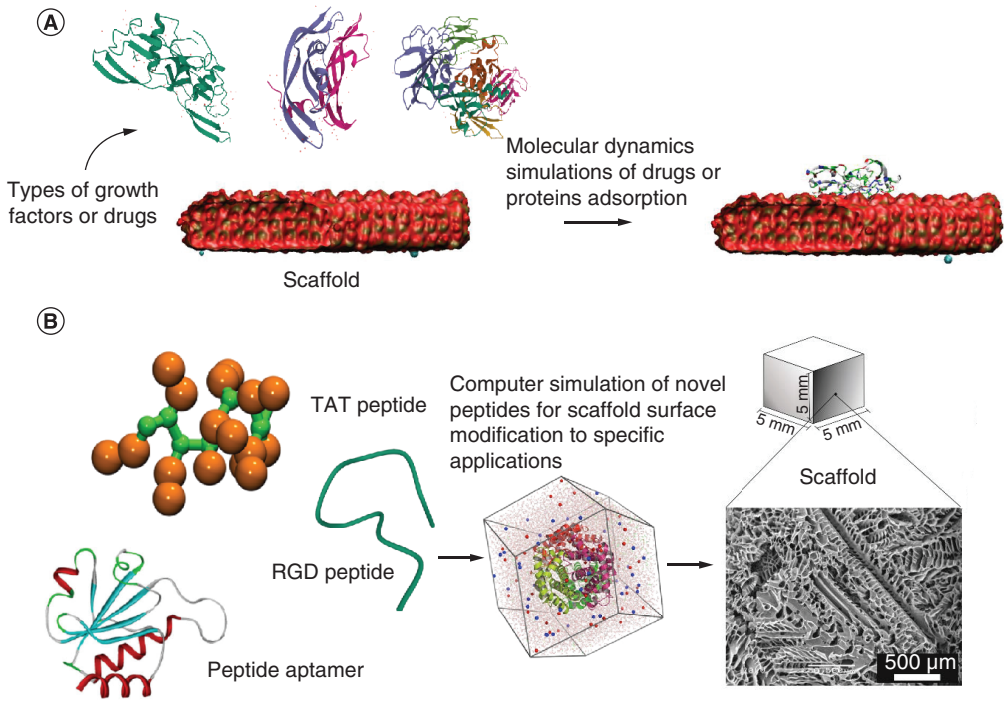

Figure 2. Applications of molecular dynamics simulation in tissue engineering. (A) Molecular dynamics (MD) simulations of adsorption and desorption of different proteins or drugs can be investigated on scaffold surfaces [66,124]. **(B)** Computational simulation of novel peptides for scaffold surface modification to the specific application. TAT: The TAT peptide (GRKKRRQRRRPQ) is derived from the transactivator of transcription that is encoded by the tat gene in HIV-1 that can penetrate cell membranes; Aptamer: Aptamers are oligonucleotide or peptide molecules that are selected to bind to specific sites on their target molecules; RGD: Arginylglycylaspartic acid (RGD) is the most common peptide motif responsible for cell adhesion to the extracellular matrix. MD simulation techniques can be used to design RGD novel peptides that bind to only one type of integrin and act specifically [129–131]. **(A)** has been adapted with permission from [66], © Elsevier (2018).

highly efficient in gene transduction; also, transgene expression can be controlled by viruses and can target specific cell types including non-dividing cells or dividing cells. Transfection refers to the process of transferring genes via nonviral vectors. Advantages of nonviral vectors include: simple manufacturing, low cost, low immunogenicity and high packaging capacity [137–140].

Current breakthroughs in gene therapy, such as CRISPR-Cas9 or iPSCs techniques along with the CRISPR-Cas9 approach, overcome the limitations of conventional gene transfer techniques. These advancements have created a new situation for gene therapy alternatives in the time is near. In fact, it is possible to regulate or correct genes *in vitro*, and consequently, abnormal cells in patients can be substituted with engineered-iPSCs [141]. In addition, another potential approach, synthetic biology, is expected to help control cell behaviors, develop new properties and enhance cell therapy capabilities, reducing the time from basic research to clinical applications [142]. Therefore, new approaches like synthetic biology and CRISPR-Cas9 can play a significant role in gene manipulation and transfer in the future in the various pathways of cell induction, cell differentiation, angiogenesis and other processes involved in the TE field.

There are some ways to safely transfer the gene in cells, including using nonviral vectors and the regulation of exogenous gene expression using expression control systems such as Tet-on/Tet-off (Figure 1C); thus, the role of synthetic biology is clearly important here. In addition, it is assumed that based on the engineered cell-mediated approach, the challenge of tissue vascularization in TE can be improved.

MD is already a valuable tool in helping to understand biology. Using MD can save cost and time and help to find new composites in TE [143]. We suppose that the careful use of MD simulations along with complementary experimental methods is a great way to make progress in TE and other fields. This possibility will only continue to expand as quicker simulations, which are also more accurate, become cheaper and more readily available.

Limitations of new biological approaches

While viral vectors are extremely effective in gene transfer, they can also cause safety concerns; including, immune reactions to viruses, limitation in packaging capacity and insertion mutagenesis [140]. Despite the belief that viral vectors pose a greater risk than nonviral vectors, nonviral vectors are less efficient in transferring genes compared with viral vectors [144]. Drug or gene transfer by non-viral vectors like nanoparticles could also have toxicity as a drawback [145].

Such as other techniques of gene therapy, CRISPR-Cas9 has drawbacks. Off-targets are still the biggest problem of the CRISPR-Cas9 approach. Since CRISPR-Cas9 was introduced in 2014, many Scientists and companies have been working on developing powerful ways to find off-targets of CRISPR-Cas9 [87,146].

Concerns about the various risks of synthetic biology are not well known; however, they mainly focus on issues including biosafety and biosecurity (such as the potential for intentional misuse of synthetic biology). Social scientists have also noted concerns related to risks, including horizontal gene transfer, disruption of ecology, unintentional exposure of humans or the environment to engineered organisms and problems related to ethical, legal and social issues [147]. Considering the risks and challenges mentioned above, optimizing and reducing the concerns of these new techniques seems necessary to continue more efficient studies in the future.

In the case of MD, the lack of representation standards, the inadequacy of optimum analysis tools, and even the complexity of storing and transmitting the massive amount of trajectory data generated are still issues that need to be resolved [148]. However, MD is already a helpful tool that contributes to a better understanding of biology.

Conclusion

This review provides an overview of research in genetically engineered cells and scaffolds for novel applications in TE, including the CRISPR-Cas9 system, synthetic biology and MD simulation. It is hoped that these genetic engineering approaches will bring us closer to therapeutic goals for humans faster than traditional methods. In recent years, many researchers have focused on the therapeutic potential of gene-modified cells; this is due to the capacity of these engineered cells to control cell differentiation, stop inflammation, manage immunological reactions and help in the repair of tissues.

The investigation of new approaches in this study will bring an efficient relationship between these approaches in the field of TE so that it can be understood in previous studies which genes play a major role in gene delivery? Then, with CRISPR-Cas approaches, these genes can be genetically engineered to increase the efficiency of cellular signaling, and based on this, efficient genetic circuits can be designed in synthetic biology to obtain intelligent and controllable tissues. Considering all these factors, it is now possible to select the best scaffold to be used for genetic engineering purposes with the MD simulation approach.

Future perspective

With novel approaches, including CRISPR-Cas, the use of new peptides (such as aptamer) to target scaffolds and the MD simulation, further advances in TE can be imagined in the future. Overall, stem cells can be a good source for genetic engineering purposes in TE; however, we expect to hear more about iPSCs for genetic engineering purposes in the field of TE in the near future. Also, with the synthetic biology approach, controllable tissues can be designed. With the CRISPR-Cas strategy, cell differentiation or immune pathways can be genetically engineered; also scaffolds with a high binding tendency can be designed with computational strategies. Using computational simulation, several proteins can be investigated for adsorption on the surface of scaffolds and the best protein obtained can be selected, and the gene of this protein can be used for the gene delivery purposes in TE; thus, the optimization process of experimental assays is facilitated. In addition, with a computational approach, the adsorption of a protein can be examined with several different scaffolds to obtain the best scaffold in terms of protein adsorption. Using the MD simulation technique, it is possible to use compounds from natural sources (such as new plant compounds) along with peptides to assess self-assembly, investigate mechanical properties and interact with various cell receptors and integrins; this approach will help a lot in getting new composites faster. The MD simulation technique can be used to examine the surfaces of scaffolds in terms of hydrophobicity or hydrophilicity and eventually find out more quickly which surfaces are more suitable for initial cell attachment and cell proliferation. It is suggested that the application of new approaches in future researches can be achieved more quickly and effectively and save costs. Although efforts to engineering cells for overexpression of specific genes are in their infancy and have a long way to reach the point of therapeutic use, they present a potentially perfect method for targeted tissue regeneration.

Executive summary

Background

- This review article provides recent advances of gene delivery and its applications in tissue engineering (TE); also, new techniques such as the CRISPR-Cas9 system, synthetics biology and molecular dynamics simulation to improve the efficiency of the scaffolds have been studied.
- The following cell engineering strategies in TE are discussed:
  - Gene-modified cells.
  - CRISPR systems and TE.
  - Other approaches of gene delivery in TE.

Scaffolds novel applications

- The topics and applications of synthetic biology in relation to TE are described in this review article.
- Understanding the applications of molecular dynamics simulations in TE can provide valuable guidelines for the future design of TE scaffolds/implants.
- Advantages of new biological approaches. This review article briefly points out the advantages of new biological techniques, including gene delivery, CRISPR-Cas9 system, synthetic biology, and MD simulation in the field of TE.
- Limitations of new biological approaches. This review article briefly discusses the limitations of new biological techniques, including gene delivery, CRISPR-Cas9 system, synthetic biology, and MD simulation in the field of TE.
- Conclusion. This review provides an overview of research in genetically engineered cells and scaffolds for novel applications in TE.
- Future perspective. With new biological approaches, intelligent scaffolds will be constructed, and further advances in TE are conceivable in the future, which may significantly contribute to clinical studies.

Author contributions

M Safaei was responsible for the overall guidance of this review. Z Abpeikar, Y Ahmadyousefi and AA Najafi were responsible for the literature collection and analysis. Z Abpeikar drafted the manuscript and AA Alizadeh critically revised the manuscript for content. Finally, all authors read and approved the final manuscript.

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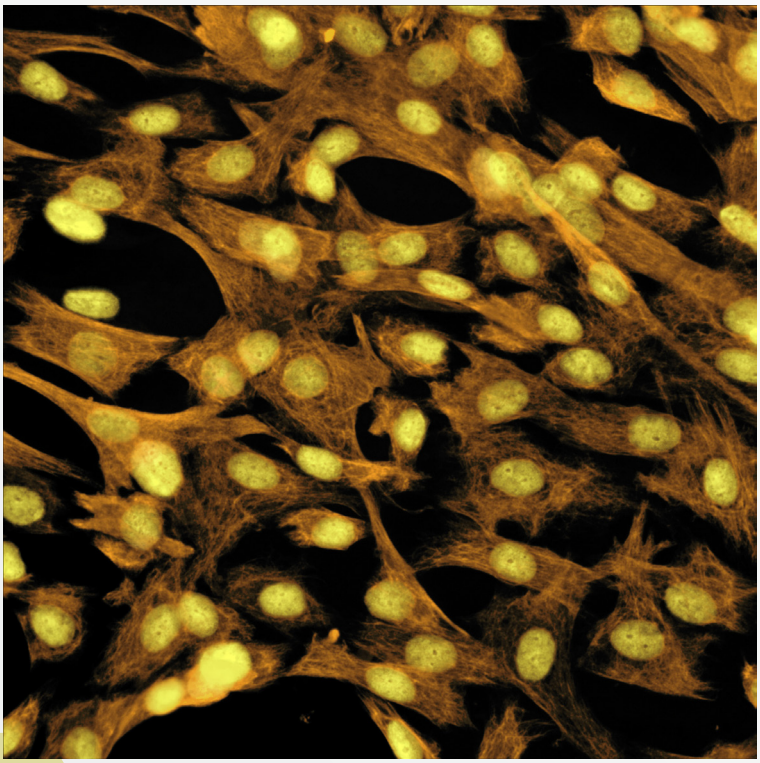

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In situ 3D bioprinting of musculoskeletal tissues in orthopedic surgery

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Annually, millions of Americans require some form of reconstructive surgery as the result of a traumatic injury, degenerative process or pathologic state. In the field of orthopedic surgery, the gold standard for augmenting bone, cartilage and soft tissue defects has been through the application of grafts, prostheses and soft-tissue flaps. Recently, there have been great advances within the field of tissue engineering including the development of 3D-bioprinting technology. Bioprinting uses biomaterials and cells to create 3D tissue-mimicking structures aimed at repairing or replacing damaged tissues. Further developments have led to *in situ* bioprinting which manufactures the tissue directly at the site of repair through handheld or portable 3D-bioprinting devices. Challenges still exist in implementing this technology. However, there is hope that one day this technology will be equipped for the operating room or clinic.

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Annually, millions of Americans require some form of reconstructive surgery as the result of a traumatic injury, degenerative process or pathologic state involving the musculoskeletal system tissues and overlying skin [1–3]. In the field of orthopedic surgery, cartilage loss, bone loss, tendon degeneration and degeneration of complex tissue interfaces such as ligament to bone are challenging to treat.

The gold standard for augmenting bone, cartilage and soft tissue defects has historically been through the application of allogeneic or autologous tissue grafts, non-custom metal prostheses and even soft-tissue flaps [4–6]. Many of these treatment options are joint-sacrificing and are unable to preserve or restore the natural biology of the pathologic musculoskeletal tissue. There are various disadvantages and complications that may result depending on the graft source, material and anatomic location.

Over the last decade, there have been great advances within the field of tissue engineering. This includes the development and implementation of 3D-bioprinting technology. Bioprinting is the process of creating 3D structures consisting of biomaterials, biomolecules and cells with the potential for creating tissue-mimicking structures aimed at repairing or replacing damaged and impaired tissues. This allows the synthetic production of biologically sound tissue configurations that mimic the native tissue cellular systems they are predicated upon [7–10]. The layer-by-layer interlacing of complex cellular architecture through the 3D-bioprinting process with biologically active scaffolds, synthetic hydrogels and extracellular matrix (ECM) proteins ultimately recreates the microenvironment of the native tissue they are intended to replace. Most recently, this technology has been translated from *in vitro* preparation to *in vivo* applications, otherwise known as *in situ* bioprinting. *In situ* bioprinting allows the fabrication of patient-specific tissues, reconstructed or printed at the defect site in a real-time clinical setting [7].

This novel, exciting, point-of-care approach offers great potential in the field of orthopedic surgery for advancing the treatment of complex musculoskeletal pathology. This review will provide a systematic examination of the most current literature on *in situ* 3D bioprinting of musculoskeletal tissues with a focus on applications to the field of orthopedic surgery and a look into the future directions of the technology.

Future Medicine

Current standard of care

The current standard of care for augmenting bone, cartilage and soft tissue defects is patient and pathology specific, however, typically involves the application of allogeneic or autologous tissue grafts or metal prostheses, all of which present their own complication profiles.

Autologous bone or soft-tissue grafts require additional dissection of healthy tissues for graft retrieval and are often associated with significant donor site morbidity and increased operative time [11–13]. A study by Younger *et al.* examining the morbidity associated with autologous bone grafts from various donor sites revealed a major complication rate of 8.6% with 3.8% requiring reoperation, 1.2% with sensory loss and 3.3% with postoperative hematomas [11]. Vinagre *et al.* outlined the disadvantages of using autologous hamstring grafts for ACL reconstruction as follows: deficits in strength following surgery, unpredictable graft quality, prolonged ligamentization of the graft and an increased risk of infection [13].

Allogenic bone or soft-tissue grafts carry the risks of potential graft rejection, immunologic reactions or disease transmission [14,15]. There are also substantial costs associated with allogeneic bone graft and bone graft substitutes as well as concern for clinical efficacy of these products [15]. The use of non-biologic or non-patient-specific orthopedic implants often result in complications due to imprecisions at the graft-defect or hardware-bone interface. Moreover, certain components of metallic implants including nickel, chrome, cobalt and bone cements may have the potential to induce allergic responses leading to implant failure, pain or the need for revision surgery [16]. The various complications associated with these restorative techniques have provided an opportunity for improvement and further research. The use of 3D-bioprinted materials as a graft substitute has the opportunity to alleviate these problems entirely and result in improved patient function and satisfaction, decrease in reoperation rates and the subsequent decreased cost of care.

Current musculoskeletal applications of 3D printing

The most of the current literature for augmentation of large musculoskeletal defects emphasizes the use of patient-specific metal implants designed for use after major trauma or extensive revision surgery [17–20]. Within the field of orthopedic surgery, the majority of this current literature is focused on the lower extremity. Kadakia *et al.* outlined the process of 3D-implant design in foot and ankle surgery where implants allow for precise anatomic restoration where autograft or allograft may not be appropriate [17]. Similarly, Steele *et al.* described the use of custom 3D spherical implants for large bony defects as an optimal alternative to traditional tibiotalaocalcaneal arthrodesis with allograft. At an average of 23 months of follow-up, the custom 3D spherical implant cohort had a significantly higher fusion rate than the femoral head allograft cohort [19]. These studies suggest that 3D-metallic implants may offer a more robust and anatomically precise solution to traditional orthopedic constructs in limb salvage when significant sized bone defects or poor bone quality are present. A study by Dekker *et al.* reported high rates of fusion, increased patient satisfaction, and improved functional outcome scores in 15 patients who required custom-designed 3D-printed cages for complex foot and ankle deformities and arthrodesis procedures [21]. At an average of 22 months postoperative, there was an 87% clinical success rate with only one infection (6.7%) and one nonunion (6.7%). While the initial results are promising, the majority of the 3D-implant outcomes published are limited to short-term follow up. However, the longevity of 3D metallic-implant success was most recently described by Nwankwo *et al.* through a single-patient case study demonstrating a successful 5-year follow up for a patient-specific 3D-printed titanium cage for a large distal tibial defect [22]. Additionally, in the field of total joint arthroplasty, custom 3D-printed implants have shown satisfactory long-term follow up. Kieser *et al.* showed a minimum 2-year survival rate for 3D custom-printed tri-flanged acetabular implants for a sizable cohort of 46 patients undergoing surgery consecutively [23].

This limited literature provides evidence that, in the setting of limb salvage, 3D-printed metal implants result in short-term satisfactory function and fusion rates. However, there is concern that the long-term success and biocompatibility may be challenged by corrosion resistance, Young's modulus and osseointegration [24,25]. The biologic–metal interface is a complicated one that often sacrifices biologic function for anatomic structure. The differential in Young's modulus of cortical bone and various metal alloys risk the formation of stress risers and weakening of adjacent bone through stress shielding [26,27]. The avascular nature of metal implants also leave them prone to infection and biofilm formation where the body is unable to adequately defend against microbial assault [28,29].

Unfortunately, metal implant technology ultimately does not take into account the secondary forces of tissue expansion, growth and deformation that occur after tissue repair. It is an unforgiving, avascular construct that may

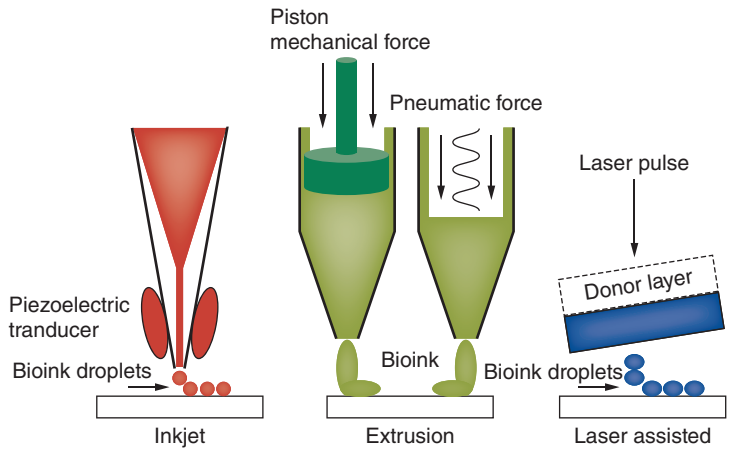

Figure 1. Example methods of 3D bioprinting.

require a more biocompatible option for improved longevity. An enhanced, biologically favorable alternative for musculoskeletal defects is through 3D bioprinted tissue [30]. These bioprinted tissues are engineered to be porous, allowing for an improved vascularized implant structure that possesses enhanced soft tissue and bony integration. This also aids in the body's ability to respond to immunologic insults. Bioprinted materials with these desirable rheologic properties allow for more precise and accurate host tissue restoration, improving upon native form and function.

Bioprinting of musculoskeletal tissues

3D bioprinting allows us to reproduce the highly organized and complex architecture of living tissue in our organ systems. In 2007, Campbell and Weis described the use of inkjet printers and related printing technologies to reconstruct and produce cells with exceptional biomimicry practical for clinical application. These first patented attempts of bioprinting catalyzed growth and innovation in the field of tissue engineering [31]. Subsequent studies have demonstrated viable skeletal muscle constructs from 3D-bioprinting platforms capable of capturing the complex architecture of myofibril bundles that allow for integration of neural fibers both *in vivo* and *in vitro* [32,33].

The main components of current bioprinting techniques include the scaffold, bioink and the cells. A good scaffold for bioinks is one that facilitate differentiation, proliferation, and migration of the biologically active cellular components and cells embedded within them. Many different cell types have been successfully used in bioprinting including fibroblasts, chondrocytes, keratinocytes, mesenchymal stem cells and osteoblasts, among others.

Bioprinting techniques primarily use hydrogels as the base material for bioinks. The high water content of hydrogels allows for cell entrapment and encapsulation without inflicting damage to the cells [34]. Bioinks are combinations of natural and synthetic materials prepared via embedding functional biomolecules within a matrix that mimics the architecture and function of a native tissue they are proposed to replace [35].

Bioinks are printed through various mechanisms including inkjet-based bioprinting, extrusion-based bioprinting, laser-assisted bioprinting and inkjet-based bioprinting (Figure 1) [1]. Extrusion-based bioprinting is simply injecting of the bioink compound through mechanical means or pneumatic compressive forces from the reservoir to a site of application with contact [1,30,36]. Inkjet-based bioprinting is a non-contact form of printing where bioink drops are generated, most commonly through thermal, piezoelectric, or electromagnetic means and transferred to the substrate. Laser-assisted bioprinting uses a laser as the energy source to deposit biomaterials onto a substrate, allowing for direct cell printing. The final major form of bioprinting, known as stereolithography, utilizes ultraviolet light to photopolymerize bioinks into patterns or fixed structure at print sites [1,37]. The primary *in situ* bioprinting techniques applicable to orthopedic surgery for bone and cartilage repair are extrusion bioprinting, co-axial extrusion bioprinting, and laser bioprinting [1,38–40].

Bioinks differ in their structural integrity and affinity for cellular adhesion among other properties. Fibrocartilage tissue is favorably created using poly (ethylene glycol) methacrylate-based bioinks, whereas, hyaline cartilage bioprinting is better served by using agarose and alginate as the base bioinks [41]. In general, the main two synthetic hydrogels in bioinks are methacrylated gelatin/gelatin methacryloyl and poly (ethyelen glycol). These require

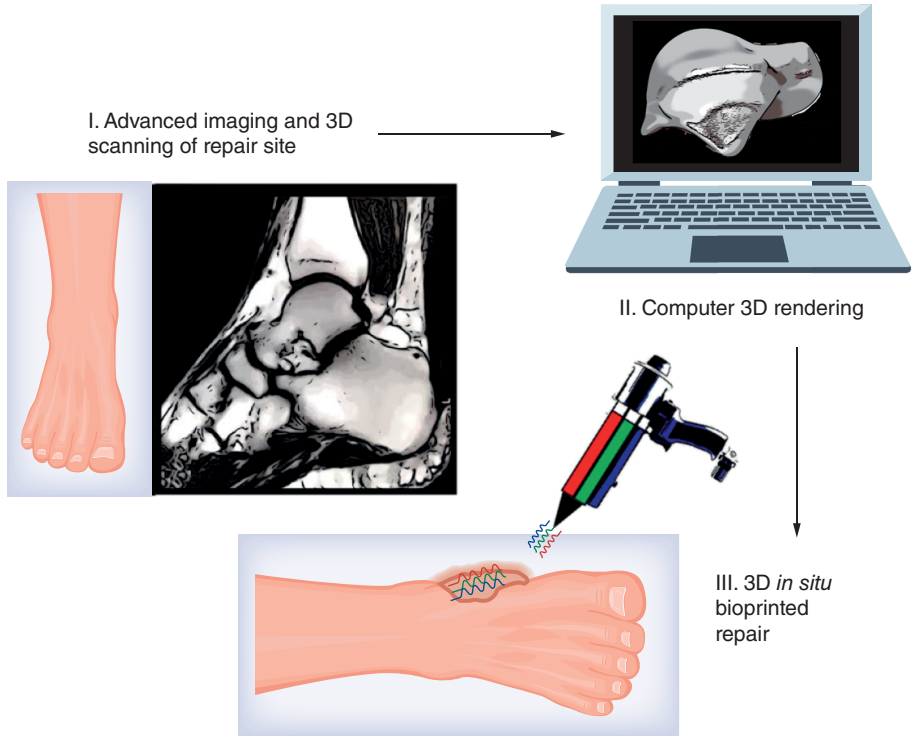

Figure 2. Process of *in situ* bioprinting.

ultraviolet light for curing which may cause cell damage, and they differ in mechanical strength with the former being stronger with slower gelation times [42].

There are various natural hydrogels used in bioinks in tissue engineering as well. They have many advantages as well as disadvantages. Although hyaluronic acid is unstable and degradable, as a hydrogel it offers excellent potential for cellular proliferation, angiogenesis and fast curing times [42]. Likewise, fibrin and gelatin allow for angiogenesis and cellular adhesion, respectively. However, they both exhibit poor stability and mechanical strength overall. When choosing a hydrogel, once must consider the balance of form (mechanical strength) and functionality because each attribute usually comes at the cost of the other. The alginate and agarose bioinks mentioned previously, are also natural bioinks which both possess good mechanical strength and durability making them excellent for hyaline cartilage bioprinting [41,42].

The theoretical schema of traditional 3D-bioprinting techniques for human tissue includes printing the tissue *in vitro* at an off-site laboratory with subsequent transportation to the patient in the clinical setting for implantation after tissue maturation. This is certainly feasible as studies have shown high culture viability and differentiation for *in vitro* bioprinted 3D musculoskeletal tendon-like tissues and articular cartilage [43,44]. However, challenges arise for bioprinting larger and more complex tissues, such as bone, which require longer culture times and specialized bioreactors to facilitate tissue differentiation and development [45,46]. These bioreactors are a primary constraint to the *in vitro de novo* progression of cellular architecture.

In situ bioprinting & bioprinter designs

In situ bioprinting, also known as *in vivo* bioprinting, takes the novel technology of bioprinting from the laboratory directly to a patient in the clinical setting. Bioinks or custom biologic prostheses, structurally engineered to facilitate both the form and function of a native tissue, are directly printed at the site of injury or tissue defect requiring repair (Figure 2). *In situ* bioprinting eliminates the aforementioned need for bioreactors given a nutrient-rich and sustainable microenvironment is available directly at the site of need. All of the required growth factors and cytokinetic components are present at the defect site during *in situ* bioprinting. The ability to engineer musculoskeletal tissues via *in situ* bioprinting confers a wealth of possibilities for the reconstruction of defects with bioactive materials that have traditionally been replaced with metallic or cellularly deficient materials.

The ability to intraoperatively print bioinks or custom prostheses directly onto a patient has multiple benefits that maximize the vitality of the implanted cells and create near-perfect fitting implants. *In situ* bioprinting enables the production of scaffolds with the precise placement of cells, biomaterials, and biomolecules into spatially predefined locations, thus eliminating implant mismatch or sizing errors. *In situ* bioprinting also substantially decreases the risk of contamination to bioprinted substrates post fabrication. It eliminates the need for sterilization post fabrication, and thus decreases the threat to cellular viability during the transport and curation process. Additionally, it allows for more efficient patient care by eliminating the treatment delay waiting for long-term culture.

There are several stages involved with *in situ* printing when computer aided modeling software is employed [1]. The first stage involves medical imaging that scans the patient anatomy and proposed site for printing application. Second, the imaging is then analyzed with computer-assisted software, and the defect site's structure is encoded into modeling software. This code is then used to create a custom-rendered 3D template for the proposed layering of bioprinted materials at the site of application [1,36].

Devices engineered for 3D bioprinting have traditionally been large-scale industrial machinery requiring a wealth of power, space, and feeder ingredients for orchestrating live, real-time 3D reconstructions [8,46]. However, *in situ* printer prototypes are being created with an increased level of finesse required for direct site application. Currently, the primary means of *in situ* bioprinting involve either the use of a handheld device or a mechanized arm [7]. The handheld versions allow providers the most freedom of use and less limitations in spatial creation, however lack the precision afforded by the mechanical arm, which runs autonomously through strict code and design. Although the mechanical arms require less direct oversight it is optimal to have an experienced controller overseeing the process to reduce the severity or frequency of any potential complications that may arise. The deciding factor, however, is generally a combination of surgeon preference, location of material deposition, and the intricacy of the design being laid down [7].

Ying *et al.* reported on a handheld, battery-powered *in situ* printing device capable of laying down bioinks at wound sites [47]. The device was ergonomically designed with an emphasis on functionality while still allowing it to be both portable and affordable with a cost of $121 USD. The design enabled control of bioink extrusion and the ability to photo-crosslink with embedded ultraviolet light emitting diodes. Similarly, O'Connell *et al.* created a biofabrication instrument deemed the 'biopen' which was capable of implanting cells with high viability potential at sites of cartilage defects [48]. Their main design objectives in creating the biopen was ergonomics and sterilizability. O'Connell *et al.*'s design had chambers for extrusion-based bioprinting controlled via a foot pedal with an attached UV light for photocuring of the delivered bioinks. These designs place an emphasis on a 'free hand' technique of application delivered by the user rather than relying on computer-assisted printing where computer navigation and software designs extrapolate the geometry of wound structures. By contrast, Ma *et al.* described the use of a robotic-arm to aid in *in situ* bioprinting of hydrogels aiding in cartilage regeneration [49]. This assistive bioprinting technology showed promising results with error rates in the printing accuracy of less than 30 μm. Their robotic-arm had a movement plane with six degrees of freedom allowing for optimal guided assistance with a self-calibrating tool for added accuracy.

All of the aforementioned studies discuss designs of novel and suitable *in situ* bioprinting devices that incorporate *in situ* repair based on pre-procedure 3D scans using predictive modeling. While they anticipate, they still lack real-time feedback mechanics with continuous 3D imaging to illustrate live geometry shifts during the procedure. This 'certify-as-you-build' strategy described by Holzmond and Li tracks the integrity of a construct layer by layer as it is printed using 3D digital-image correlation [50]. Following the printing of a single layer, stereoscopic imaging is taken. The current print layer is scrutinized by 3D digital-image correlating technology in comparison to the anticipated model. Any significant difference demonstrated between the live and predicted model is flagged. A handheld bioprinter using navigation or robotic-arm assistance could be capable of improving these real-time, flagged printing errors.

Musculoskeletal applications of *in situ* bioprinting

Various literature has shown *in situ* bioprinting's applicability in the field of orthopedic surgery for intraoperative bioprinting to aid in repair [32,33,38–40,49,51]. Traditionally, cartilage injury and osteochondral defects have been managed with joint-sparing procedures including microfracture, mesenchymal stem cell therapies, osteochondral allograft transfers as well as joint-sacrificing procedures including arthrodesis and arthroplasty [38,52,53]. Outcomes following these joint-sparing treatment options have yet to demonstrate restoration of the innate biomechanics and durability of articular joint hyaline cartilage. In 2010, Cohen *et al.* was among the first to explore the additive

manufacturing technology for *in situ* repair of osteochondral defects. The authors used artificially induced lesions in two *ex vivo* bovine femur specimens, and CT scanning was used to generate a 3D path for *in situ* repair [51]. They used an alginate hydrogel to repair the cartilage lesions and a novel demineralized bone matrix formula for bone repair. Their post-print geometric analysis revealed acceptable error rates for the bioprinting based on acceptable criteria from clinical literature. Osteochondral and chondral repair prints had less than 0.1 mm printer error rates which was within the benchmark of the study. This study was the first to show the potential for accurate *in situ* repair of bony and chondral surfaces alike that may be introduced in the operating room after preoperative planning with consideration for the intended application sites.

Bony lesions were also investigated by Keriquel *et al.* in 2010 when they demonstrated the potential for *in situ* repair and printing using high-throughput biologic lasers in mice calvaria defects [40]. This preliminary data supported the use of *in situ* applications using nano-hydroxapatite for defect repair. They later reported use of an improved and biologically favorable construct of mesenchymal stromal cells and nano-hydroxapatite for *in situ* repair of mice calvaria defects in a single flat plane [39]. Using laser-assisted bioprinting, repairs were made and x-ray microtomography was used to analyze mice calvaria at 1 and 2 months post printing. Their study not only showed sufficient healing and integration of bony defects based on different bioprint patterning, but also the laser-assisted bioprinting had no significant detriment to the underlying cerebral tissue. Keriquel *et al.* and Cohen *et al.* illuminated on *in situ* bioprinting to bony defects applicable to orthopedics and demonstrated *in vivo* viability and integrative capability of the bioprinted tissues containing either hydrogel bioinks or live mesenchymal stromal cells [39,40,51].

Further studies by Ma *et al.* demonstrated *in vivo* cartilage repair and regeneration using robotic-assisted *in situ* bioprinting techniques with hyaluronic acid methacrylate (HAMA) as a bio-ink [49]. A total of twelve New Zealand rabbits were divided into three groups all with International Cartilage Research Society (ICRS) grade IV trochlear groove osteochondral defects surgically produced. One group served as a control while the other two underwent repair of the defects. The *in situ* group had HAMA bioprinted *in situ* at the defect site directly using a robotic-arm assisted device. The other had the defects repaired with manual hydrogel implantation. The regenerated cartilage in the hydrogel implantation and *in situ* 3D-bioprinting groups demonstrated no difference in the biomechanical and biochemical properties, validating the therapeutic efficacy of the *in situ* bioprinting for osteochondral defects in an animal model. They concluded that the bioprinting promoted cartilage regeneration and did not impair hyaline cartilage regeneration at the trochlea defect.

Li *et al.* reported successful *in situ* bioprinting in other orthopedic injury models [38]. Bone and cartilage defect models of *ex vivo* specimens were artificially created including an ICRS grade IV osteochondral defect, a long bone segmental defect, and a femoral condyle fracture. The defects were 3D scanned and computer-assisted design techniques were used to encode the geometries of the bony and cartilaginous defects present. They utilized two different photopolymerized hydrogels, an alginate hydrogel for bony and osteochondral defects, and a modified sodium hyaluronic acid hydrogel for cartilage defects. Both bioinks and the bioprinting processes were shown to satisfactorily repair all three defect types in the *ex vivo* models created. In one of the first live animal models, Di Bella *et al.* reported striking evidence supporting *in situ* bioprinting to aid in the early regeneration of cartilage in living sheep [54]. Full thickness chondral defects were made on the weight-bearing surface of the lateral and medial femoral epicondyles of each extremity in six mature male sheep. The defects were treated with one of four treatment methods including biopen 3D *in situ* application of bioink and mesenchymal stems cells, pre-constructed bench-based bioscaffolds, microfracture, and an untreated control group. Following blinded macroscopic and microscopic evaluation of the different treatment groups, the *in situ* 3D-printed bioscaffold repairs showed improved characteristics and structural integrity compared with the other three cohorts. Di Bella's sheep study and Ma *et al.*'s rabbit study are among the only available literature comparing 3D *in situ* bioprinting techniques to other treatment standards [49,54].

Most recently, Mostafavi *et al.* utilized a composite scaffold for reconstructing bone defects that utilized osteo-conductive hydroxyapatite [55]. This composite was different from Keriquel *et al.*'s mice calvaria defect study in that it combined polycaprolactone and zinc oxide for antibacterial properties [56,57]. They used a handheld melt-spun 3D-printing device to print the scaffold directly at the site requiring application. Human mesenchymal stem cells were cultured *in vitro* on the hydroxyapatite/polycaprolactone/zinoxide bioprinted scaffolds and showed osteoblast viability and differentiation. Additionally, *in vivo* implantation showed neo blood vessel formation when implanted into subcutaneous folds of mice that was dose dependent on increasing composition of hydroxyapatite in the scaffold [55].

Table 1. Associated challenges in applications of 3D-bioprinted materials *in situ*.

Challenges for *in situ* bioprinting
Ethical considerations for use of embryonic stem cells in an *in vivo* setting
Host integration of the bioprinted material at the recipient tissue site Immune response to bioprinted materials and scaffolds Angiogenesis and vascularization of bioprinted materials Sterilization of bioprinted materials without disrupting molecular integrity of bioprinting cells and scaffolds Proliferation and differentiation of bioprinted cells following administration *in situ* Curing and gelation of the bioprinted product and surrounding tissue compatibility with curation process
Costs associated with *in situ* bioprinting: Composing biologic constructs and stem cells for bioprinting 3D scans of recipient sites for donor bioprints
Intellectual property and overall rights to usage of bioprinters, stem cells and bioprinted scaffolds

Challenges in *3D in situ* bioprinting

There are various challenges that *in situ* printing techniques must overcome before becoming an accepted treatment option for orthopedic surgeons in real time (Table 1). Traditional bioprinting requires large-scale industrial printing machines and space for these procedures to take place. The portability and mobility of bioprinters for use in the operating theater will require machines agile enough to navigate not only the site of printing but also the surrounding clinical environment. Additionally, sterile processing of these machines will be of concern. Most procedures requiring *in situ* bioprinting will be intraoperative in nature, requiring a heightened level of antisepsis. This same principle of sterility applies for the bioinks, custom prosthesis, and the polymerization processes introduced at the target print site as well. A high sterile burden will be required for all *in situ* printing procedures.

For specific repair sites, the overall access and adequate surgical exposure of the *in situ* printing surface's sophisticated contours and tissue planes may present a challenge. Additionally, the tensile forces and deformation properties and blood and body fluid components need be appreciated for their effect on procurement and cross-linkage of active biologic components within bioinks. The application of bioink and substrate material can be tenuous and tedious owing to the unstable infrastructure of the bioactive printed materials before polymerization, and intervening substances may further decrease integrity during initial application [7].

Both form and function must be considered. When repairing an anatomic site, the replacing prosthesis or tissue may exhibit the form, but it is also essential that the function is restored. A study by Kim *et al.* demonstrated human muscle progenitor cells and human neural stem cells aid in the integration of host nerve tissues when combined in bioprinted constructs at large muscle defects in rodents [32,33]. The addition of neural stem cells allowed for restoration of function that otherwise may not have been possible with larger segmental muscle defects. The function of the underlying tissue should be in consideration when *in situ* printing large defects.

The post-printing maturation of the tissue construct is a significant challenge warranting further investigation. The maturation and survival of the tissue depends on factors such as structural fidelity, nutrient and oxygen supply, immunogenic response, cells proliferation and differentiation, and degradation kinetics of the construct [7]. Scaffold vascularization and incorporation at the existing site, particularly with the insufficient supply of nutrient and oxygen to deeper tissues due to the absence of an internal vascular network, is a prerequisite in the tissue maturation process which must be further addressed.

Bioprinting cellular layers in scaffolds without well vascularized networks or the ability to create a nutrient and oxygen rich environment will result in clumping of cells and necrotic bioprint tissue centers. Studies exist showing the ability to create conduits and networks capable of supporting nutrient and oxygen trafficking. Miller *et al.* bioprinted synthetic tissue with vascular networks composed of carbohydrate glass in a vascular casting approach. The vascular casts allowed for adhesion and lining of endothelial cells. This endothelization of a vascular network within synthetic bioprinted tissue was able to sustain pulsatile blood flow and ultimately metabolic activity in rat hepatocytes [58]. Likewise, Dolati *et al.* also showed the ability to create stable bioprinted vascular conduits that were encapsulated with smooth muscle cells of coronary artery tissue [59]. These conduits were over a meter long and utilized alginate hydrogels with a backbone of carbon nanotubes to allow for metabolic activity in human coronary artery smooth muscle tissue. Applying this technology into large-scale tissue constructs still requires more investigation until this is applicable clinically.

Additionally, muscles need to contract and retain a level of tissue compliance similar to the surrounding anatomy for adequate function. Other surgical fields exploring *in situ* bioprinting strategies may offer insight on how to

optimize functionality of bioprinted tissues in orthopedics. Various techniques have been utilized to develop tissues and organs for implantation including blood vessels, cardiac tissues, cornea, and skin, all requiring a high level of tissue compliance. Cardiothoracic and vascular surgery studies have utilized tissue engineering strategies for cardiac myocyte regeneration and in the augmentation and repair of intimal vessel tissues that often require much greater elastic dimensional properties and expansile characteristics [60–62]. Knowledge gained from these fields may better serve the application of orthopedic associated bioprinted tissues necessitating contractile properties and in handling joint reactive forces. While the field has made significant advances, fabricating large and complex structures with multiple cell types remains a challenge. Last, there are cost, ethical, and regulatory considerations that must be addressed before on-site constructing or repairing of tissues and vital organs becomes clinically available.

Future directions in orthopedics

Despite the existing challenges, there is the need and utility for *in situ* bioprinting which has the potential to significantly advance regenerative musculoskeletal medicine and improve patient outcomes. First, advances in device design and software are warranted to ensure a device that can navigate the clinical setting, maintain sterility, is minimally invasive, ergonomic, has freedom and range of motion to allow printing on a dynamic surface, and is user-friendly. The majority end-users of *in situ* bioprinting techniques will be surgeons that are not well versed in the engineering complexities of the devices. Thus, it has been proposed to incorporate artificial intelligence into the designs to ensure precision and surgical quality [7].

Second, innovations in bioinks are required. In order for a bioink to be both printable and viable *in situ*, it must possess a number of mechanical and biological characteristics that will provide structure but also allow for the integration of host tissue and the eventual overtake of host cells from the printed stem cells as the scaffold is resorbed over time [7]. Next, advanced, high-resolution, scanning systems are required to understand the complex 3D anatomy of the defect to allow for robotic-based bioprinting. A developing technology that compliments 3D printing and allows for both increased patient safety and overall advancement in the potential of *in situ* bioprinting is portable 3D-scanning devices. These scanners allow for quick and accurate measurement of potential lesions and bioprinting targets that can then be transmitted for real-time printing, without the need of expensive and potentially harmful pre-operative imaging. Additionally, real-time scanning allows for the identification of potential errors and the ability for adjustments to be made during the *in situ* printing process, if a scan needs to be re-done or additional scans performed [38]. As the quality and portability of portable 3D scanners and software continues to develop, as well as a reduction in cost associated with increased market competition, their use for *in situ* bioprinting is sure to increase.

A final, and arguably most important, aspect of the *in situ* printing process is assuring that the bio ink is viable throughout the printing process. To do so, real-time monitoring of the tissues while they are in the printing device and as they are laid down as part of the printing unit itself or an associated device will assure that the newly printed tissues remain viable as they settle into the host environment. This is ideally achieved through a series of sensors monitoring cell viability parameters and feedback mechanisms aimed and identifying and removing devitalized bio ink and early identification of environments that are not suitable for bio ink deposition [7].

Despite the many nuances at play with this technology there are many benefits. The primary advantages of and rationale for *in situ* bioprinting include improved time to repair without extended culturing of engineered tissues *in vitro*, decreased risk of contamination through direct application without the need for transport, sufficient growth medium directly at the host injury site among adjacent tissues, and ultimately improved 3D templating of repairs and alterations with direct visualization of defects in live time while bioprinting.

Conclusion

The expansile and continuously progressive field of tissue engineering and 3D *in situ* bioprinting has promise for restoring anatomical form and improving function for orthopedic surgery patients. At the present, portable 3D bioprinter prototypes have shown potential for restoring skeletal muscle defects, osteochondral lesions, and deficiencies in bone. There are unique challenges in the translation of 3D bioprinting from benchside to bedside. Significant challenges still exist for *in situ* processes including the high sterile burden required for *in situ* bioink substrates, prostheses, and bioprinting machines; the need for printers to be intelligent and mobile enough for precise surgical instrumentation; consideration of tissue geometries, deforming forces, and expansile properties; and consideration of blood and other bodily fluids that may offer a challenge in the cross-linkage and polymerization of 3D-printed products. Future directions within orthopedics include advances in tissue engineering, device design

and software, bioinks, preoperative or intraoperative 3D-scanning systems, and real-time quality assurance via integration of artificial intelligence, machine vision, inspection sensors, and feedback control systems. Despite the existing challenges, there is the need and utility for *in situ* bioprinting which has the potential to significantly advance regenerative musculoskeletal medicine and improve patient outcomes.

Future perspective

Three-dimensional printing technology has already been used to create structurally sound, non-biologic implants in the field of the orthopedics with great success. However, biologic integration of 3D bioprinted tissues with the structural integrity of current metallic implants and longevity is still on the horizon. Reconstructive orthopedic surgery relies on transferring sterile implants and/or products to the field of application. Future directions for bioprinting include the ability to restore and repair large cartilage or musculoskeletal defects directly at the site *in vivo*. The promise is that one day, these bioprinted materials will fully incorporate with host tissues and further vascularization would allow for even more sophisticated molecular mimicry. We speculate the next steps in tissue engineering through *in situ* 3D bioprinting will allow for the direct administration of bioprinted material by surgeons in the operating room to augment and repair large musculoskeletal defects. Advancements in the field of molecular genetics may allow for induction of host genetic machinery by the bioprint to allow for synergistic growth, expansion, and ultimate incorporation of the 3D bioprinted tissue.

Executive summary

- Annually, millions of Americans require some form of reconstructive surgery for large musculoskeletal tissue defects. There is potential for 3D bioprinting *in situ* to play a major role in helping repair or augmenting these defects.

Current standard of care

- The current standard of care for augmenting bone, cartilage, and soft tissue defects is patient and pathology specific, however, typically involves the application of allogeneic or autologous tissue grafts or metal prostheses, all of which present their own complication profiles.

Current musculoskeletal applications of 3D printing

- The field of orthopedic surgery currently utilizes 3D-printed non-biologic and metallic implants for reconstruction procedures.
- The 3D-printed implants currently being used show adequate implant survival long-term, patient satisfaction and improved functionality.

Bioprinting of musculoskeletal tissues

- 3D bioprinting allows us to reproduce the highly organized and complex architecture of living tissue in our organ systems.
- The main components of current bioprinting techniques include the scaffold, bioink, and the cells.
- Bioinks are printed through various mechanisms including inkjet-based bioprinting, extrusion-based bioprinting, laser-assisted bioprinting, and inkjet-based bioprinting.

***In situ* bioprinting & bioprinter designs**

- *In situ* bioprinting, also known as *in vivo* bioprinting, takes the novel technology of bioprinting from the laboratory directly to a patient in the clinical setting 3D tissue is created at the site of injury.
- Bioprinter designs include handheld devices that are portable and robotic-assisted machines to aid in *in situ* bioprinting.

Musculoskeletal applications of *in situ* bioprinting

- Various literature has shown *in situ* bioprinting's applicability in the field of orthopedic surgery for intraoperative bioprinting to aid in repair including: cartilage repair, osteochondral lesions and bone defects in animal studies.

Challenges in 3D *in situ* bioprinting

- Host integration of the bioprinted material at the recipient tissue site.
- Costs associated *in situ* bioprinting.
- Intellectual property and overall rights to usage of bioprinters, stem cells and bioprinted scaffolds.

Future directions in orthopedics

- Artificial intelligence in guiding *in situ* bioprinting.
- Navigation and robotic assistance integrated into user experience.

Conclusion

- Portable 3D-bioprinter prototypes have shown potential for restoring skeletal muscle defects, osteochondral lesions, and deficiencies in bone.
- Unique challenges exist in the translation of 3D bioprinting from benchside to bedside.

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One or more authors have financial and conflict of interest disclosures including: consulting for Stryker, Conventus, RTI, Restor3d, Extremity medical, Orthofix, Ossio and Artelon. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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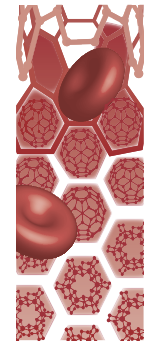

Scaffold biomaterials and nano-based therapeutic strategies for skeletal muscle regeneration

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Skeletal muscle is integral to the functioning of the human body. Several pathological conditions, such as trauma (primary lesion) or genetic diseases such as Duchenne muscular dystrophy (DMD), can affect and impair its functions or exceed its regeneration capacity. Tissue engineering (TE) based on natural, synthetic and hybrid biomaterials provides a robust platform for developing scaffolds that promote skeletal muscle regeneration, strength recovery, vascularization and innervation. Recent 3D-cell printing technology and the use of nanocarriers for the release of drugs, peptides and antisense oligonucleotides support unique therapeutic alternatives. Here, the authors present recent advances in scaffold biomaterials and nano-based therapeutic strategies for skeletal muscle regeneration and perspectives for future endeavors.

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Skeletal muscle dysfunction & potential use of tissue engineering

Skeletal muscle is the most abundant tissue in the body. This tissue exerts contraction-dependent mechanical functions, such as movement, body support, breath and endocrine functions [1]. Muscle damage that produces alterations of mechanical tasks can be generated by denervation, toxic myopathy, myositis, sarcopenia, cachexia, trauma and genetic defects [2,3]. In the context of injury, skeletal muscle activates its repair system. A process divided into three phases: degeneration, regeneration and remodeling [4].

In the first phase, there is a rupture of muscle tissue that alters fibers, blood vessels and nerves [5]. Consequently, the sarcolemma is damaged, favoring the entry of calcium ions and activating hydrolases and proteases, leading to tissue necrosis and the release of inflammatory cytokines [6–8]. Together with the cellular debris produced by the damage inflicted on the muscle, these cytokines activate resident immune cells and recruit peripheral immune cells to assist in the regeneration process [9]. In the second phase, signals released by damaged muscle activate satellite cells, a population of quiescent stem cells with myogenic potential located between the basal lamina and the sarcolemma [10]. Once activated, satellite cells proliferate and migrate to the site of injury. At this point, the satellite cells are transformed into myoblasts. In the third phase, the myoblasts differentiate into mature myocytes, fuse and form multinucleated myotubes that integrate into damaged muscle fibers to repair muscle tissue [11]. At this stage, the regenerated muscle is revascularized and reinnervated. Resident fibroblasts generate connective

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tissue that transiently replaces the necrotized myofibers. Further, new myofibers substitute for fibrotic scar tissue, concluding the muscle repair [12]. Several mechanisms can regulate muscle regeneration. In recent years, it has been demonstrated that extracellular vesicles (EVs), a heterogeneous population of membrane-enclosed nanoparticles released from cells, can modulate muscle regeneration when they are secreted by mesenchymal stem cells [13,14]. In addition, muscle regeneration could be tracked by EV secreted during the myogenic process [15].

The regenerative process just described corresponds to the most common and abundant muscle fibers, which are called extrafusal fibers. In addition, skeletal muscle contains intrafusal fibers, which are part of the muscle spindle, a structure directly involved in proprioception [16]. Interestingly, proprioception is impaired in aging, frailty, diabetes and neuromuscular diseases such as multiple sclerosis, Parkinson's disease, muscular dystrophy and peripheral nerve injuries [17–19]. The regeneration of intrafusal fibers maintains a similar pattern to that of extrafusal fibers. However, this process also has several unique features, such as increased satellite cell numbers and sustained expression of myogenic factors associated with early muscle development in the muscle spindle [16]. To study the mechanism involved in the repair of intrafusal fibers, *in vitro* methods are needed. In this context, intrafusal fibers engineered in a 3D and aligned microenvironment should result in a more efficient differentiation and mature phenotype [16,20]. Indeed, to recapitulate the connections between intrafusal fibers and nerve fibers, a support that could consist of a nano-based platform such as 3D-hydrogel is required [16].

Despite the fact that muscle can regenerate, in several pathological conditions or in the case of accidents, muscle injuries provoke volumetric muscle loss (VML), resulting in at least 20% of muscle mass loss. In these situations, the rate of fibrotic tissue formation exceeds the differentiation and maturation of the myoblasts so that a thick nonfunctional scar obstructs the fusion of the myotubes [12,21]. Therefore, patients affected by a VML condition cannot restore muscle mass due to impaired regeneration [22]. Therapeutic strategies for VML, based on a healthy muscle graft's autologous transplantation, have been assayed. However, they have been inefficient because they cannot restore muscle function due to fibrous tissue accumulation [12,23]. Thus, the absence of effective therapy to enhance the muscle repair system and the lack of treatment for VML have encouraged tissue engineering (TE) for the purpose of muscle formation [24]. TE strategies have been developed and tested to improve skeletal muscle regeneration. In brief, tricultures of myoblast, embryonic fibroblast and endothelial cells seeded on 3D porous polymers were found to improve vascular density in an intramuscular implant on quadriceps muscle [25]. The transplant of predifferentiated myogenic cells was also shown to improve new vessel formation [26]. Regarding innervation, muscle cells cocultured with nerve cells in a 3D construct of fibrin gel increased muscle differentiation and maturation concomitant with increased force generation using *in vitro* and *in vivo* models [27,28]. Furthermore, a synthetic scaffold enriched with the promyogenic factor IGF-1 was implanted in the tibialis anterior muscles of rats, acting as a delivery system for the myogenic factor and enhancing recruitment of myoprogenitor cells, differentiation capability and muscle formation [29] These antecedents confirm that TE strategies can improve the recruitment, proliferation and/or differentiation of myoprogenitor cells and the vascularization and innervation of muscle using various biomaterials, which will be described later [30].

On the other hand, genetic muscle diseases also represent a significant challenge due to ineffective treatments. Duchenne muscular dystrophy (DMD) is the most common disease among these pathologies, a congenital condition characterized by dystrophin gene mutations that initiate degeneration and regeneration cycles with chronic inflammation, fibrosis and atrophy [3]. Current pharmacological therapy for DMD includes treatment with glucocorticoids and, for those with certain specific mutations, antisense oligonucleotides aiming to induce exon skipping and dystrophin production [31]. None of these treatments is capable of completely reversing the adverse effects of DMD. Due to the inability of current strategies to treat the aforementioned skeletal muscle disorders, novel procedures that involve the application of nanomaterials with tremendous clinical potential as scaffolds in TE or nanocarriers for the release of oligonucleotides and anti-inflammatory drugs, among others, have emerged [24,32,33].

Foundation of tissue engineering based on polymeric materials

In the 1980s, Langer and Vacanti described how diverse tissues and organs, including the liver and intestine, could be developed using mammalian cells seeded on 3D scaffolds of biodegradable and biocompatible polymeric materials. When these devices were implanted in animal models, the novel tissues and blood vessels had a permanent functional activity. Together with these findings, it was also discovered that the 3D scaffold's configuration was essential to mimic natural fractal branching patterns, which allowed the survival of functional tissue devices [34]. Later, Langer and Vacanti published their article (cited over 4000 times), '*Tissue Engineering*', in *Science*, which was the foundation for the field [35]. In this work, TE was defined as '*an interdisciplinary field that applies the*

principles of engineering and the life sciences toward developing biological substitutes that restore, maintain, or improve tissue function' [35,36]. At present, leading researchers in the field state that the aim of TE is the development of tissue and organ substitutes for maintaining, restoring or augmenting the functions of their injured or diseased counterparts *in vivo* [37]. The main limitations are the lack of renewable sources of functional cells that can be immunologically compatible, the lack of appropriate biomaterials with desired mechanical, chemical and biological properties and the inability to generate extensive vascularized tissue that can easily integrate into the host and that suits the architectural complexity of native tissue [37].

Several disciplines, such as biology, biomaterials and bioengineering, have contributed to the knowledge and development of TE [38]. Strategies for generating new tissue include biologically active scaffolds that induce a regenerative response, consisting of cells capable of self-assembly, delivery of soluble signals and the manufacture of a complex matrix with 3D architecture that mimics the topographic context of the tissue to regenerate [38]. New scaffolds with biodegradable materials have been developed in search of biocompatibility and bioactivity to improve the proliferation and attachment of the cells seeded on this junction via angiogenesis [34].p

Despite 30 years of scientific progress in TE, very few engineered tissues, including skin, ear and blood vessels, have been translated into clinical application [38]. The skin was the first engineered tissue to reach clinical application through seeded cells with the potential to generate skin in scaffolds produced with natural biomaterials such as collagen, cellulose and chitosan, or with synthetic biomaterial [39,40]. In the ear, TE is based on chondrocytes seeded on synthetic biodegradable polymers with the shape of an auricular structure [41,42]. Regarding blood vessels, the most common TE approaches are extracellular matrices, cell sheets and degradable synthetic scaffolds [43,44]. In these three cases of regenerated tissues, the synthetic polymers most commonly used are polyvinylpyrrolidone, polyethylene-glycol (PEG), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly-L-lactic acid, poly(ε-caprolactone) (PCL) and poly-4-hydroxybutyrate, and copolymers generated by a mixture of these polymers [40,42,44]. Recent advances in nanomaterials for skeletal muscle regeneration include the use of various additives in scaffolds that provide constructs that are responsive to electrical, magnetic and photothermal stimulation [21].

Tissue engineering for skeletal muscle

The materials chosen for the TE scaffold are essential factors regardless of the type of device developed. Polymeric materials such as natural or synthetic polymers are commonly used because they are biocompatible and biodegradable, provide adequate mechanics and guide cell differentiation [45]. Natural polymers such as glycosaminoglycans (GAGs) are essential components of the skeletal muscle extracellular matrix (ECM). They can be combined to form a platform that mimics the muscle ECM environment and cellular interactions. Due to their biocompatibility and biodegradability, the most studied GAGs for TE are hyaluronic acid and chondroitin sulfate [46]. On the other hand, synthetic polymers, such as poly(lactic-co-glycolic acid) (PLGA), PEG and PCL, provide mechanical resistance that resembles that generated by collagen [47], allowing cell proliferation and byproducts degraded over time to be absorbable by the surrounding cells [48]. The regeneration of neuromuscular interaction is a crucial factor in skeletal muscle recovery. Some components, such as graphene and polyaniline (PANI), enhance muscle regeneration due to the conductive properties that guide the orientation of the myoblast and the elongation of the resident neuronal tissues [49]. TE strategies mainly focus on implant development to reconstruct damaged tissue. These strategies involve regenerative tissue and scaffolds that allow functional recovery.

In recent years, advancements in TE have included the use of nanomaterials in the manufacture of new scaffold structures with conformation that improves TE's regenerative properties, achieving clinical success in ear, blood vessel and skin regeneration [38,50]. In particular, TE strategies to form skeletal muscle consider myoprogenitor cells seeded on a nanostructure-like scaffold built with biomaterials of diverse origin that mimic the microenvironment and allow an appropriate chemical and physical stimulation (see Figure 1) [51]. Numerous studies *in vitro* have shown the successful growth of skeletal muscle within these scaffolds, while other assays also have determined the recovery of functionality *in vivo*. However, only a handful of analyses have exhibited muscle repair in patients with VML [52–54]. The success of nanomaterials in TE depends in part on the nature of the inflammatory responses they evoke. The immune response elicited by nanomaterials varies from neutrophil infiltration to macrophage recruitment and ultimately to fibrous capsule formation. Those reactions are mainly driven by the molecular mechanisms that orchestrate the integration or rejection of nanomaterials in the tissue [55]. The persistent activation of acute inflammatory pathways involves the complement system, Wnt and TGF-β signaling, among others [56]. Inadequate inflammation in response to nanomaterials after muscle injury could affect muscle regeneration because

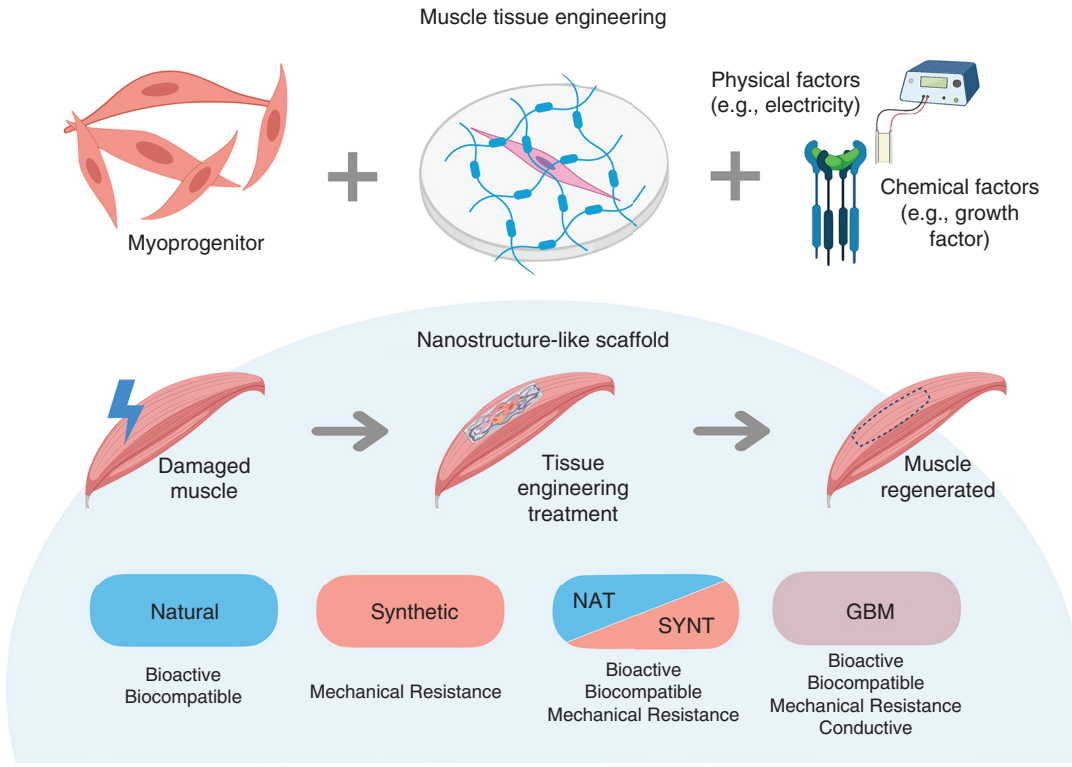

Figure 1. Nanostructure-like scaffold composition applied to tissue engineering for skeletal muscle regeneration.
The main components of skeletal muscle tissue engineering are myoprogenitor cells, nanostructure-like scaffolds and physical/chemical factors. Combining these three components generates a graft implanted in a damaged muscle to promote its regeneration. Nanostructure-like scaffolds can be manufactured from biomaterials of various origins, such as NAT, SYNT, hybrid (nat./synt.) and GBM. Each biomaterial has advantages that contribute to the application of tissue engineering: bioactivity, biocompatibility, mechanical resistance or conductivity.
GBM: Graphene-based material; NAT: Natural; SYNT: Synthetic.

it is dependant on the inflammatory response mainly orchestrated by macrophages. The macrophage inflammatory status controls the proinflammatory and proresolutive stages that conclude with a regenerated muscle [57].

The innate and adaptive immune response must be considered for each regenerative initiative that involves the use of nanomaterials because the interaction with the immune system can potentially lead to immunosuppression, hypersensitivity, immunogenicity and autoimmunity due to the inherent physical and chemical nanomaterial characteristics. The cell-mediated adverse immune response could be mediated by lymphocytes or dendritic cells, which could even trigger platelet activation [58–60].

The use of nanoparticles also represents a unique alternative for treating genetic muscle diseases (Figure 2). Gene-editing technology, combined with nanocarriers as drug-delivery systems, could be the most suitable alternative for the systemic administration and targeted delivery of therapeutic molecules [61]. Nanocarrier-based drug delivery comprises the technological approaches of nanostructures loaded with therapeutic structures in sizes ranging from 1 nm to 100 nm. The most studied nanocarrier systems are nanoparticles, nanocapsules, nanoemulsions and nano-sized vesicular technologies [32,33,62]. These strategies maximize molecules' bioavailability by increasing selectivity, site-specific targeting, solubility or even half-life [63]. Nonetheless, no clinical studies have been performed on skeletal muscle; however, *in vitro* and *in vivo* assays demonstrated high selectivity of nanocarriers for skeletal muscle and a proper capacity to diminish skeletal muscle dysfunction [64–67].

TE strategies for skeletal muscle have three main components: nanostructure-like scaffolds, made with biomaterials from natural or synthetic origin, or even a combination (hybrid materials); progenitor muscle cells; and chemical (e.g., growth factors) and physical (e.g., magnetic fields, mechanical stretching) stimulating elements [51]. Although natural or synthetic polymers are the central biomaterials used in TE, carbon-based materials are the nanomaterials chosen to build hybrid scaffolds [68]. An example is graphene, which has attractive properties favorable for muscle regeneration [69]. The use of scaffolds made with natural, synthetic or hybrid biomaterials fulfills the template

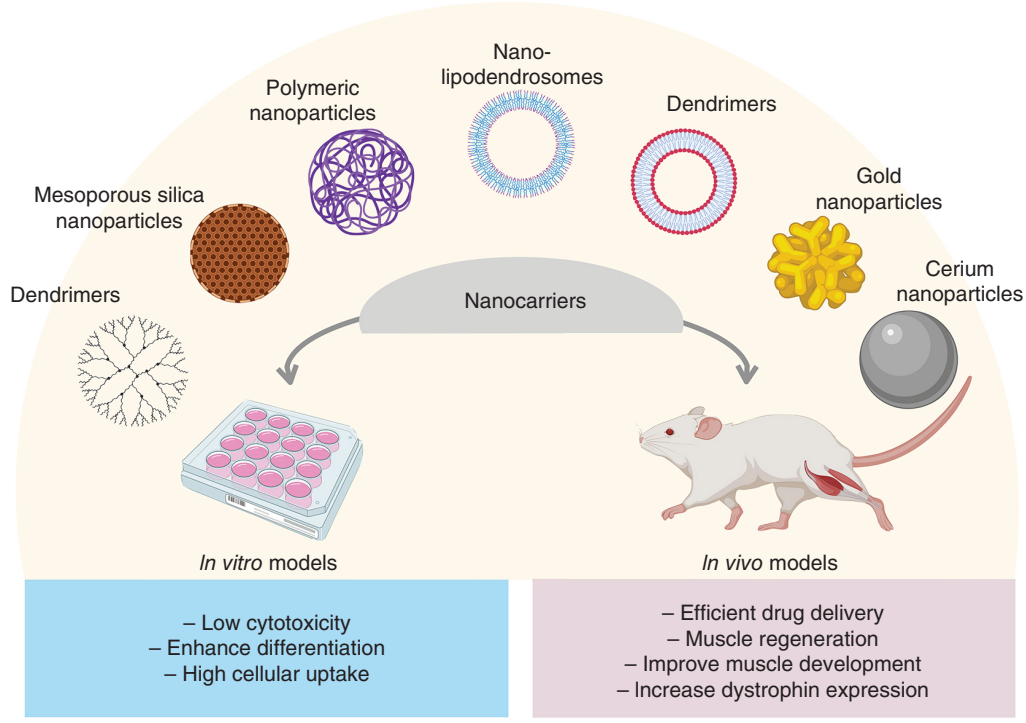

Figure 2. Nanocarriers used as drug-delivery systems in skeletal muscle. Several nanoparticles (dendrimers or polymeric-, silica-, gold- and cerium-nanoparticles) have been used as drug-delivery systems for skeletal muscle. Reported *in vitro* advantages are low cytotoxicity, improved differentiation and high levels of cellular uptake. Also, *in vivo* models have suggested that nanoparticles improve muscle development and regeneration and are efficient drug-delivery systems via the production of increased muscle protein expression.

matrix's function. Cells with myogenic potential and growth factors can be incorporated to enhance muscle tissue repair [24]. Details of the studies carried out for the different types of nanomaterials are presented in subsequent sections. The discussion section will focus on the nanostructure-like scaffold's material: natural, synthetic and hybrid scaffolds in the skeletal muscle regeneration area.

Scaffold-based natural biomaterials

Skeletal muscle is a highly anisotropic structure supported by its ECM, a complex mesh containing collagens, proteoglycans, glycoproteins and elastin. ECM is indispensable because it provides mechanical support to muscle fibers, nerves and blood vessels. The interaction between myoblast, muscle fiber and ECM involves several biological processes, such as embryogenic development, growth, skeletal muscle differentiation and repair [70]. In this context, the use of ECM components as natural biomaterials has been adopted since the beginning of TE development to exploit its biocompatibility and favor cellular processes such as proliferation and differentiation [21,35]. In particular, the use of these natural biomaterials has been widely assessed in strategies of skeletal-muscle repair based on cell therapy with collagen, alginate, laminin, fibrin, hyaluronic acid, chondroitin sulfate and decellularized ECM (dECM) from skeletal muscle as structural support to transplanted cells [21,46,71,72].

Collagen is the most widely used ECM component because it favors myogenic differentiation, increases biocompatibility with the host and improves biomaterial bioactivity [73,74]. *In vitro* studies using 3D, collagen-based scaffolds demonstrated a correct alignment of the muscle cells within these platforms [30,75,76]. Other studies using a murine model of muscle injury showed that myoblasts seeded in the collagen gel scaffold, which was further implanted in skeletal muscle, repair the muscle by fusing the myoblasts with the pre-existent muscle tissue [77]. Developing a collagen scaffold combined with another ECM component was also found to improve muscle hypertrophy and functional recovery in a VML murine model [78]. Although collagen scaffolds provide an excellent platform for muscle cell growth, some properties, such as scaffold strength and conductivity, can be improved to optimize its function, increasing the sensitivity to mechanical and electrical stimulus, which is essential to maintain muscle activities [79,80].

In 2007, Cornwell *et al.* designed a scaffold based on fibrin microprocesses aligned in the longitudinal axis of the muscle containing growth factors [81]. The implantation of this scaffold in conjunction with a human myogenic cell line in the tibialis anterior achieved a 90% force recovery in a VML mouse model. Nevertheless, the native muscle fibers exhibited an apparent decrease in the deposition of connective tissue that forms the scar and fibers, which were not correctly aligned [82].

Scaffolds based on hydrogels have also been developed with natural biomaterials, including a particular mixture of thiolate hyaluronic acid and thiolate chondroitin sulfate. *In vitro* experiments using the C2C12 myoblast seeded in this hydrogel revealed correct proliferation, differentiation and expression of myogenic markers. Even more significant was the notable effect in a murine quadriceps VML model. The hyaluronic acid/chondroitin sulfate scaffold enhanced migration of satellite cells, myofiber formation in conjunction with angiogenesis and innervation after four weeks of implantation [46].

The use of dECM from skeletal muscle was determined to be superior to other types of hydrogel since it recapitulates the native tissue's ECM composition and bioactivity and mimics the complex structure of skeletal muscle ECM [83]. A porcine diaphragmatic dECM-derived hydrogel has been used as an excellent scaffold for repairing diaphragmatic muscle presenting appropriate biomechanical properties, high biocompatibility and adaptability *in vivo* [84]. In addition, porcine muscle dECM-derived hydrogel can also be mimicked by 3D bioprinting, in which muscle cells align and differentiate with a high degree of myotube formation [85].

Although the results obtained with scaffolds based on hyaluronic acid thiolate hydrogels and chondroitin sulfate thiolate are promising, they exhibit a rapid degradation and low mechanical resistance when transplanted, which limits their broad applications in muscle regeneration over time [40]. Some chemical modifications of these scaffolds improved their resistance to degradation and mechanical features; however, the broad differences between the manufacturing sources caused limitations concerning reproducibility and use of those platforms [86]. Despite natural scaffolds presenting excellent bioactivity and high biocompatibility, as shown in Figure 1, several disadvantages, including their mechanical weakness, inconsistency fabrication and rapid degradation, have lead to the development of novel strategies to overcome these shortcomings.

Scaffold-based synthetic biomaterials

Artificial platforms in muscle regeneration arise from the need to compensate for the deficiencies of natural scaffolds. The primary materials employed to prepare scaffolds based on synthetic biomaterials include PGA, PEG, PLA, PCL, polyurethane (PU) and polypropylene (PP) [24,87]. These scaffolds can be formatted in different nano-configurations, such as foams, hydrogels, electrospun scaffolds and meshes [24].

Hydrogels are the most widely used formats for muscle regeneration. The elaboration of hydrogels has the main objective of obtaining a structure and composition similar to healthy skeletal muscle [88]. The use of PEG-based hydrogel offers excellent biocompatibility in skeletal muscle [89]. Thus, satellite cells cultured with PEG hydrogel improve their proliferation and differentiation *in vitro* and enhance graft acceptance *in vivo* [90]. The advantages of synthetic hydrogels are their increased resistance and mechanical affinity, compared with those of natural origin, and their adaptation to the structure and shape of the tissue [89]. However, scaffolds formed by synthetic hydrogels have less bioactivity than scaffolds of natural biomaterials, mainly displayed as low cell adhesion resulting in deficient tissue formation due to the nonspecific interactions between the platform and the muscle cells [88].

Cell alignment is one of the most critical factors for optimal muscle regeneration and this is achieved through scaffolds of nanofibers generated by electrospinning. Mesh nanofibers are commonly used for skeletal muscle regeneration and they provide excellent structural support. *In vitro* studies using electrospun meshes show that myotubes align to differences with myotubes formed in a standard differentiation medium [74,87].

As indicated in Figure 1, another hallmark of synthetic scaffolds is their high reproducibility in several manufacturing procedures as well as their ease of manipulation, flexibility, mechanical resistance, slow degradation and high precision in the tissue's physical, chemical and structural properties [24,74,87,91]. Studies based on scaffolds manufactured with synthetic materials indicate that platforms can trigger an adverse immune response, causing fibrous capsule formation. However, most adverse effects were found to be generated by the chemical nature of biomaterials instead of the scaffold structure or the device [73,74].

The 3D-printed scaffolds can provide an environment that aids the growth and differentiation of myoblast cells. Polymers such as PCL, PLGA, PVA and PU are commonly used to give the stiffness and topology needed to orientate the myoblast's fibrils. For example, Cheng *et al.* developed a 3D scaffold using PLGA as a material source for the electrohydrodynamic jet-printing process. The generated 3D platform presented a gap of 50 μm that conferred

enhanced cell adhesion and proliferation compared with a 2D system. In addition, the C2C12 cells cultured in this 3D scaffold exhibited an increased differentiation of myoblasts [92]. Such 3D scaffolds highlight significant advances in transplant devices that attempt to improve VML injuries.

Synthetic biomaterials are also used to recover peripheral nerve conductivity. One study has examined the design of nerve guidance conduits (NGCs) based on reduced graphene oxide and gelatin methacrylate hydrogels. The authors speculated that NGCs' mechanical and electric properties explain the outstanding capability to promote axon regrowth, myelinization, muscle and nerve regeneration in a murine model. Further, NGCs' degradation profile and biocompatibility make them a suitable alternative to autograph therapy [93].

Hybrid natural–synthetic scaffolds

Natural–synthetic hybrid scaffolds are fabricated with natural and synthetic biomaterials [94]. Their mixture achieves a dual, complementary, combined and synergic effect. It maintains the advantage of the bioactivity's natural components to promote muscle regeneration and the benefits of synthetic materials' mechanical and physical properties (Figure 1) [94]. Hybrids scaffolds have been designed in several formats: synthetic nanofibers encapsulated in ECM-based hydrogel, covers derived from natural components on a mesh of synthetic materials and synthetic–natural-based electrospun compounds [95]. PU electrospun nanofibers embedded in ECM hydrogel were found to exhibit flexibility, elasticity, mechanical sustainability of ECM components as well as enhanced bioactivity [96]. Notwithstanding these benefits, its effects on myogenesis or muscle regeneration have not been determined [97,98]. The use of PCL/chitosan electrospun nanofiber scaffolds has shown a better alignment and differentiation of myoblasts than PU alone [99]. In addition, the use of PCL/chitosan nanofibers supplemented with soluble factors, such as Wnt3a protein, showed increased differentiation of human embryonic myogenic cells *in vitro* [52].

One particular arrangement for the hybrid scaffold is the microchannel. This disposition provides alignment and guidance for seeded myoblasts. PU microchannels with fibroin silk or gelatin increase the hydrophilicity of platforms and improve cell adhesion properties, proliferation rate and formation of aligned myotubes [100]. However, the best effect on muscle function has been reported by implanting muscle cells seeded in a PCL/collagen electrospun nanofiber scaffold in a mouse model of an injured diaphragm. In this pathological model, the diaphragm's histological features and mechanical properties were recovered [53]. Another variable to improve the activity of collagen scaffolds was the incorporation of electrically responsive polypyrrole. This scaffold showed a fivefold increase in conductivity and increased myoblast differentiation and myotube maturation in a C2C12 cell culture [80].

Indeed, the combination of natural and synthetic materials for developing hybrid scaffolds has benefits compared with the separate use of these materials. In this way, the fabrication of hydrogels, microchannels and electrospun nanofibers improves mechanical and physicochemical properties and increases *in vitro* bioactivity (Figure 1) [94]. Notably, *in vivo* studies have demonstrated the recovery of the mechanical properties of skeletal muscle [53]. Consequently, the use of natural–synthetic hybrid platforms provides a possible application for restoring skeletal muscle mass in patients with VML.

Some studies have shown the formation of aligned myotubes on an electroconductive nanopatterned substrate based on highly aligned collagen bundles, which were formed by capillary force lithography. Interestingly, the electroconductive substrate showed the upregulation of myogenic regulatory factors Myf5, MyoD and myogenin, which play significant roles in myogenic differentiation and maturation, indicating that substrate conductivity could play an essential role in engineering functional and biomimetic skeletal muscle tissues [101].

In addition, recent research has found evidence that a bioink material based on gelatin methacrylate (GelMA) and laponite particles release VEGF. The rational design of this bioink is its application together with a handheld printer that photopolymerizes the GelMA *in situ* over the injured muscle. The *in vivo* trials of a murine model showed that the bioink provided a sustained VEGF release over the affected region on skeletal muscle. Furthermore, on the treated subject, the sustained release of VEGF was linked with a twofold enhancement of mechanical properties and reduced fibrosis compared with untreated subjects. This strategy aims to improve the first aid over a VML lesion, such as in military trauma care [102]. At present, 3D-cell printing technology based on a dECM bioink is gaining great interest as a means to produce 3D tissue for medical applications. Kong *et al.* developed a 3D neuromuscular tissue using a porcine central nervous system tissue dECM bioink. The resulting device allowed the culture of neurons and muscle cells for over a month. The neuromuscular junction generated in this model exhibited a higher innervation of muscle tissue cells and a better electrical contractile response than the models based on Matrigel [103].

A recent study examined a scaffold based on human skeletal muscle cells and neuronal stem cells, mimicking the neuromuscular junction present in skeletal muscle. In this initiative, the assembled scaffold improved the

differentiation and survival of bioprinted cells in a murine model of tibialis anterior muscle defect. The constructed platform also enhanced the muscle's innervation capacity and proliferation, supporting the successful applications of hybrid materials [104].

Scaffolds containing graphene-based material

In recent years, graphene has emerged as an innovative nanomaterial with attractive properties for application in TE. Graphene has a hexagonal lattice structure that reaches a thickness of less than 0.5 nm. It is an excellent electrical and thermal conductor and is mechanically robust and light-absorbing [105]. However, graphene precipitates in biological media due to its high hydrophobicity, which decreases its biocompatibility for TE [106]. Graphene oxide (GO), a graphene derivative, contains many hydroxyl groups on its surface, turning it into a highly hydrophilic material. These properties transform it into a material with high biological value due to its large surface and chemical composition [107]. GO's features allow it to adsorb protein and other molecules interacting with cellular components, increasing cellular attachment [69]. Also, GO can be incorporated into materials commonly used in TE, given its electrical conductivity, mechanical resistance and hydrophilicity (Figure 1) [108]. Thus, GO incorporation increases the bioactivity of scaffolds commonly used in TE. GBM hybrid scaffolds are formed by combining graphene, or any of its derivates, with a natural or synthetic polymer. The principal aim of this mixture is to complement the properties of each material. The methods used to produce GBM hybrid scaffolds are hydrogels, electrospun fibers, meshes and 3D-printed foam [69].

Regarding hydrogels, research has shown that they induce improved myogenic differentiation. A hydrogel fabricated of horseradish peroxide-reactive gelatin polymer containing GO improved myogenic differentiation without affecting cellular proliferation [109]. A graphene-polysaccharide hydrogel enhanced the scaffold's wettability, electrical conductivity and tensile strength properties, and increased the myoblast spread and myogenesis [110]. A conductive hydrogel composed of reduced GO and polyacrylamide improved myogenic gene-marker expression under electrical stimulation [111]. In addition, incorporating micropatterns into the GO and polyacrylamide hydrogel allowed the differentiation of aligned myoblasts, enhanced myogenesis under electrical stimulation and improved the correct implantation *in vivo* [112]. Similar results were obtained with a poly(citric acid-octanediol-polyethylene glycol)-graphene electroconductive nanocomposite [113]. Another system used in GBM is foam. A laminin-coated nickel/graphene foam showed high muscle differentiation and electrical stimulation *in vitro* [114]. Also, PU/GO foams allowed spontaneous myoblast differentiation (attributed to the community effect), improved cell communication and facilitated the necessary signals for fusion and differentiation [115].

Nanofiber scaffolds generated by electrospinning have been used to correctly align the muscle progenitors for optimal muscle differentiation and regeneration [71]. In this manner, graphene incorporation added features to this structure, such as strength, flexibility, optical transparency and conductivity [116]. With the use of GO/PU electrospun nanofibers, graphene also increased bioactivity, which was reflected by the rise of adhesion, spreading, proliferation and myoblast differentiation [108]. A GO and PCL electrospun fiber mesh correctly differentiated and aligned human cord blood-derived mesenchymal stem cells to skeletal muscle cells [117]. The graphene concentration, which proportionally influences the myogenic potential, must be considered for manufacturing and using graphene-based strategies such as electrospinning for muscle TE [118]. The use of GBM composed of synthetic and natural materials has also been studied. Electrospun fibers of PLGA and collagen impregnated with GO significantly enhanced the adhesion, proliferation and differentiation of C2C12 myoblasts [115,119]. However, GBM studies using 3D printing for TE in skeletal muscle are not currently available. Palmieri *et al.* used 3D-printed scaffolds (without GBM) for muscle regeneration [69]. The authors suggested that graphene incorporation would enhance the results obtained from the studies they analyzed by adding the features previously described to the 3D-printed scaffolds.

Indeed, incorporating graphene (and its derivatives) into natural, synthetic and hybrid scaffolds improves their properties. Thus, the development of hydrogels, foams, meshes and electrospun nanofibers with GBM enriches physicochemical, mechanical and physical properties useful for muscle TE (Figure 1). Moreover, GBM incorporation into these scaffolds enhances the bioactivity (an increase of adhesion, spread, proliferation and differentiation of myoblasts) *in vitro* with the biocompatibility of grafting *in vivo*. GBM hybrid scaffolds are a promising strategy with high therapeutic potential for use in skeletal muscle regeneration, particularly in patients with VML.

Nanocarriers for skeletal muscle regeneration

Nanocarriers comprise a diverse group of technological approaches at a nanometer scale, ranging from 1 nm to 100 nm, that could have biodegradable properties and represent an emergent drug-delivery system [32,33,62]. The research has focused on developing site-specific and selective delivery (targeted delivery) based on nanotechnology, maximizing the bioavailability of molecules from chemicals, proteins or even genes. The most common nanocarriers are nanoparticles, nanocapsules, nanoemulsions and nano-sized vesicular technology, the structure of which essentially depends on the formulation method [62,63]. There are some features of the nanocarrier-based drug-delivery system that are considered advantageous compared with conventional therapies. For example, nanocarriers have increased bioavailability due to their small size, which allows them to pass through endothelial cell gaps. Nanocarrier systems can also increase hydrophobic drug solubility and prolong the half-life of its cargo. These properties are considered valuable in improving the pharmaceutical properties of the drug and off-target effects [33,120–122]. Interactions between nanoparticles and their target cells depend on several factors. Among them are the chemical and physical properties of the delivery system. Therefore, it is also necessary to consider nanoparticles' influence on the proliferation rate and cell membrane features of muscle cells [123,124]. Determining the interactions of nanocarriers with muscle cells to improve them is crucial in developing new and promising biomedical nanocarrier-based strategies [124–126]. Despite the fact that most of these properties of nanocarrier-based drug formulations have been investigated in several diseases, such as cancer and cardiovascular diseases, for skeletal muscle, few studies using and describing *in vitro* models have been reported [33,127,128].

Dendrimers are well-defined nanostructures composed of a central core and branched, functionalized units characterized by high stability and loading capacity as drug-delivery systems. They have been widely studied and found to release peptides and deoxyribonucleic acid (DNA), among other therapeutic molecules [63,129]. In terms of skeletal muscle, studies were performed with hydroxyl-terminated poly(amidoamine) dendrimer as a carrier for angiotensin (1–7) (Ang-(1–7)), a peptide with an antiatrophic effect that presents high instability under oral and intravascular administration. The results demonstrated a highly antiatrophic effect in terms of muscle strength, fiber diameter, myofibrillar protein levels and atrogin-1 and MuRF-1 expressions in a murine model [130]. Moreover, hybrid drug-delivery platforms based on dendrimers and liposomes (nanolipodendrosome) conjugated with glatiramer (an anti-inflammatory compound) and MyoD (a myogenic protein factor) showed a significant effect in the induction of mass muscle increment in various foot muscles without side effects [129]. Those reports suggest that dendrimer and dendrimer-conjugated systems could promote muscle mass restoration and function in different animal models with a low dosage, thus being a beneficial and efficient drug-delivery system (Figure 2).

Dendrimers and other drug-delivery platforms could be critical for gene therapy and treating dystrophic diseases [63]. In recent years, restoring dystrophin synthesis in DMD has been assayed by delivering antisense oligoribonucleotide (ASO). ASO is a synthetic, single-stranded short DNA-like molecule (15–30 nucleotides) with the ability to modulate alternative splicing to induce exon skipping and exon inclusion [131]. Specifically, for DMD, ASO allows splicing of the dystrophin pre-mRNA transcript, restores the translation reading frame and rescues dystrophin expression. In dystrophic mice, ASO has been used to recognize the splice donor of dystrophin exons 23, 51 and 45–53, allowing the correct expression of dystrophin [132,133]. In humans, clinical trials have demonstrated that ASO administration significantly increases exons 45, 51 and 53 skipping, concomitantly with increased dystrophin protein expression [134–136]. Despite these promising results, the use of ASO has limitations due to the poor systemic distribution and possible nephrotoxicity observed with high doses of other ASOs [131]. Several nanocarrier systems have successfully improved ASO administration in a dystrophic mouse model [63,137].

The use of nanocapsules based on poly(methyl methacrylate) surrounded by cationic groups and functionalized with ASO has exhibited good distribution and internalization rates in different types of muscle. It also induces restoration of dystrophin expression in the heart, diaphragm, gastrocnemius and quadriceps muscles in dystrophic *mdx* mice [138]. A different surface modification combined with alginate improves the delivery of an oligoribonucleotide parenterally administrated. This antecedent suggests that nanocapsule surface modification can enhance skeletal muscle affinity but cannot solve the delivery inefficiency when oral administration is used [137,139]. Other polymeric-mediated delivery systems have been developed based on cross-linked polyethyleneimine [140,141]. Those strategies successfully deliver morpholino oligomers *in vitro* and *in vivo*, with low toxic effects and dependent on molecular size and hydrophobic properties (Figure 2). Moreover, intravenous administration in a dystrophic *mdx* mice model demonstrated that this nanocarrier system increases dystrophin expression on the tibialis anterior, quadriceps, diaphragm and biceps muscle [121,141]. Those results indicated that nanocarrier systems could help pre-

vent ASO's protection and slow release in dystrophic animals, favoring a low dose required to produce a functional effect (Figure 2). A disadvantage of nano-based delivery systems is the possibility of altering cell viability or having toxic effects. A clear example of nanocarriers and adverse effects is the copper nanoclusters used to diagnose some diseases [142–144]. In this case, copper nanoclusters induce a decrease in the C2C12 cell viability due to oxidative stress, reduced mitochondrial membrane potential and produced atrophy in mice [145].

Gold-based nanoparticles (AuNPs) have been described as an efficient vector for cell transfection characterized by high cellular uptake, low cytotoxicity and the potential for modification [146,147]. The positively charged colloidal AuNP has fewer cytotoxic effects and more capability for gene delivery than conventional cationic nonviral vectors or lipofectamine-based delivery, showing high biocompatibility and enhanced transfection efficacy with nontransfectable cells such as myotubes [64,148]. AuNPs may have advantages over other metals (i.e., large surface area per unit mass), and have been used to investigate interactions between nanoparticles and cells. Drugs conjugated to AuNP have shown an efficient delivery system by allowing a low dosage to induce biological effects [65,149,150]. There is evidence that AuNPs and gold/silver nanoparticles (Au/AgNPs) favor myogenic differentiation in muscle cells. Treatment with AuNP and Au/AgNP-based hydrogels is also associated with skeletal muscle regeneration *in vivo* [66]. AuNPs can also be used to create complexes with organic compounds to improve their biological effects. AuNP and gold–heparan sulfate treatment enhanced muscle development in a chicken embryo model, showing only minimal muscle structure distortion [149]. The complex of AuNPs with IL-4 was found to induce high immune cell infiltration and increase muscle fiber area and contraction force in an ischemic injury of the tibialis anterior muscle mice model [65]. These antecedents support the idea that AuNPs have a potential role in promoting muscle regeneration, which could be induced directly on muscle cells and modulate the extracellular milieu, serving as an efficient delivery system (Figure 2).

Mesoporous silica nanoparticles (MSNPs) are characterized by a large loading capacity, chemical surface functionalization and excellent biodistribution properties [32,151]. Some studies have shown that MSNPs are internalized and can release their content inside the muscle cells, suggesting they provide a controlled delivery system [151,152]. MSNP film is also biocompatible with cultured myoblasts, keeping its capacity to increase proliferation and enhance myotube formation (Figure 2) [151,152]. Despite the benefits, dissolving silica's potential release could have adverse side effects [32,151].

Another versatile system used for improving muscle regeneration is based on liposomes, which are defined as vesicles composed of a phospholipid bilayer enclosed in an aqueous cavity. The ability to modify the liposome surface allows the analysis of different coating properties and their effect on muscle cells [62,126]. For example, polydopamine-coated liposomes can be used as a drug-delivery system because of their interaction with myoblast cells and their stability in physiological conditions [153]. Moreover, PEGylation-modified liposomes, a modification that reduces adhesion to plasma proteins increasing its circulatory concentration, diminishes the internalization in myoblast cells compared with non-PEGylated liposomes [32,126]. These results indicate that it is necessary to analyze the interactions of cells with modified delivery systems to improve physicochemical properties. Recent research also showed that PLGA nanoparticles interact with primary human myoblasts, and further, they are endocytosed without side effects. These nanoparticles are more biocompatible than MSNPs and liposomes, which might induce adverse effects [33].

Cerium oxide (CeO_2) and perfluorocarbon have also been used to successfully synthesize nanoparticles to induce improved biological effects in skeletal muscle. CeO_2 can form a nontoxic antioxidant nanoparticle and improved diaphragm contractibility in a rat peritonitis model associated with lesser sarcolemma damage [154,155]. Moreover, perfluorocarbon nanoparticles conjugated with rapamycin are an effective treatment for dystrophic *mdx* mice by inducing improved physical performance and muscle strength [67]. These results confirm that diverse nanoparticle systems can improve muscle function in mechanical and genetic animal models of pathologies, either as nanoparticles alone or in conjugation with drugs (Figure 2).

Interestingly, the use of PEGylated liposomes might alter the viability of human myoblasts [33]. Despite the extensive acceptance of PEGylated nanoparticles as a safe system, PEG coating in other types of nanocarriers also has disadvantages. For example, PEG-AuNP has no adverse effects on C2C12 myotube viability, but some modifications to the metabolic cell profile and more susceptibility to cell death induction have been reported [147]. PEG seems to provide a defective coat material for skeletal muscle tissue independent of the nanocarrier system. Comprehending nanocarriers' interactions with skeletal muscle cells through analyzing the internalization rate or delivery properties is a priority in optimizing nano-based drug-delivery systems. Although some materials have shown positive effects in skeletal muscle cells, the inherent limitations of *in vitro* studies suggest the need to perform

more analyses using *in vivo* models to describe the impact of innovative delivery systems directed to promote muscle regeneration and restore muscle function.

Strategies based on nanoparticle platforms can improve muscle regeneration by directly affecting the muscle cell or modeling the extra- and intracellular milieu by delivering available drugs. Some nanocarriers can be used as DNA-delivery systems to treat genetic conditions such as DMD. In addition, optimized and more efficient drug delivery will enable nanosystems to treat dystrophic diseases and VML conditions more efficiently. However, more molecular studies are necessary to understand the modulation and toxic effects of nanocarriers.

Conclusion

Skeletal muscle injuries from VML or genetic diseases such as DMD currently have no treatment. The use of nanomaterials-based strategies to generate new skeletal muscle by TE is a promising therapeutic option. Natural, synthetic and hybrid scaffolds have been assessed to improve altered muscle regeneration or cell therapy. These methods present advantages but also certain disadvantages that must be further analyzed and overcome. The most promising strategy thus far assessed is GBM incorporation into scaffolds because it enhances bioactivity with high therapeutic potential to be used in skeletal muscle regeneration and patients with VML. Alternatively, for DMD or other genetic muscle diseases, nanocarriers can be used as DNA-delivery systems to improve the release and systemic distribution of oligonucleotides or anti-inflammatory drugs used in treatment. In addition, 3D-printing scaffold technology generates novel avenues to enhance tissue regeneration, muscle innervation and conductivity. The application of decellularized scaffolds as a platform is a tremendous approach that will provide novel insights.

Future perspective

To a large extent, TE studies have mainly focused on developing nanomaterials, but more studies to evaluate the benefits for skeletal muscle must be designed and conducted. Most studies have been limited to assessing muscle regeneration, accompanied by muscle strength recovery. However, there are relevant molecular parameters that have not been considered. It would be interesting to evaluate if the type of muscle fibers generated by TE is the same as those of the receptor muscle where the scaffold was implanted. This could help determine whether ECM scaffolds can be developed using primary cultures or isolated fibers from muscles that share metabolism with muscles affected by VML. This option can facilitate the generation of oxidative or glycolytic fibers, depending on the muscle's need to be regenerated [156]. Independent of the type of muscle metabolism, this is a high demand on tissue *per se*, and it must be innervated for its contraction. In this regard, approaches have emerged based on muscle constructs relying on host vessel infiltration and constructs that are prevascularized *in vitro* before implantation [157]. Similarly, constructs have been developed that allow correct innervation [157]. These strategies could be probed in conjunction with nano-strategies that favor muscle innervation and vascularization. GBM also presents promising results and could improve the vascularization and innervation of novel tissue generated by TE.

More than 60 therapeutic nanoparticles have been approved by the FDA or the EMA, with a significant proportion being liposomes focused on oncology treatment. However, there is an extensive gap in research regarding the broad array of diseases that potentially could be treated using nanosystems, including skeletal muscle diseases [158]. From the perspective of neuromuscular diseases, gene therapy has progressed. In 2017, the FDA approved Eteplirsen, an ASO that increases dystrophin in skeletal muscle, to treat DMD [159–163]. Unfortunately, Eteplirsen has to be administrated intravenously every week, but a sustained-release platform based on nanoparticles could provide significant benefits for patients with DMD. In particular, the frequency and administration route of drugs are critical for quality of life in the pediatric population.

In the case of promising peptides with antiatrophic and antifibrotic effects, novel nano-based delivery systems, such as dendrimers, could be assayed in DMD models. AuNPs have been demonstrated as excellent materials for developing and optimizing innovative drug-delivery systems to small molecules, have a high affinity to skeletal muscle and lack adverse effects. Since AuNPs have already been evaluated in clinical studies to prove its safety profile and effectiveness, a future clinical trial could be performed to evaluate the treatment of VML or DMD [164,165].

We are in a new era of muscle regeneration TE, in which bio- and nanomaterials contribute as adjuvants to innovative approaches that combine and manipulate the biological systems as raw materials for new technologies, including the 3D bioprinter and bioinks based on dECM. Nanomaterials with high conductivity, such as graphene, are expected to play a significant role in creating functional muscle tissue that presents conductivity and innervation. The experimental evidence supports significant advances in TE with natural, synthetic and hybrid biomaterials for muscle regeneration. The use of nanocarriers also provides novel therapeutic strategies for gene therapy and release

drugs and inhibitors. Nevertheless, further exploring the interactions and the molecular mechanisms involved in the cellular and tissue response demands novel endeavors.

Executive summary

Skeletal muscle dysfunction & potential use of tissue engineering

- Inefficient strategies to treat skeletal muscle disorders with altered regeneration or loss of muscle mass require the development of novel procedures applying nanomaterials as scaffolds in tissue engineering or nanocarriers for the release of treatments.

Scaffold-based natural biomaterials

- Natural scaffolds present excellent bioactivity and high biocompatibility to generate skeletal muscle but involve mechanical weakness, inconsistency in fabrication and rapid degradation.

Scaffold-based synthetic biomaterials

- Synthetic scaffolds have high reproducibility from different manufacturing sources, easy manipulation, flexibility, mechanical resistance, slow degradation and high precision. However, they have less bioactivity than scaffolds of natural biomaterials and could cause some inflammatory immune responses.

Hybrid natural–synthetic scaffolds

- Hybrid scaffolds present high bioactivity and generate recovery of mechanical muscle properties.

Scaffolds containing graphene-based material

- Graphene-based material incorporation into scaffolds enhances bioactivity with high therapeutic potential to be used in skeletal muscle regeneration and in patients with volumetric muscle loss.
- Novel approaches based on 3D-printing scaffold technology generate unique alternatives to improve tissue regeneration, muscle innervation and conductivity.

Nanocarriers for skeletal muscle regeneration

- Some nanocarriers can be used as DNA-delivery systems to treat genetic conditions such as muscular dystrophy.

Future perspectives

- Bio- and nanomaterials contribute to combine and manipulate biological systems as an essential material for dECM created by 3D-bioprinters and bioinks.
- Bio- and nanomaterials combine and contribute to manipulate the biological systems as an essential material for dECM created by the 3D-bioprinter and bio-inks.

Author contributions

F Tacchi: writing the original draft and visualization; J Orozco-Aguilar: writing the original draft and visualization; D Gutierrez: writing the original draft and visualization; F Simon: writing, review and editing and funding acquisition; J Salazar: writing the original draft; C Vilos: conceptualization, writing the original draft, review and editing and funding acquisition; C Cabello-Verrugio: methodology, conceptualization, writing the original draft, review and editing, funding acquisition, visualization, supervision and project administration.

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Key trends in engineering tissues for regenerative medicine

For our Spotlight on tissue engineering, we surveyed our audience to find out the latest trends, opinions.

What is the focus of your work?

- 47% Basic research/discovery
- 28% Therapeutic product manufacturing
- 21% Process/product development
- 4% Quality assurance/control

What therapeutic area(s) do you work in, if any?

- Neurological 24%
- Dental 8%
- Respiratory 4%
- Cardiovascular 20%
- Gastrointestinal 16%
- Dermatological 4%
- Musculoskeletal 20%
- Osteological 4%

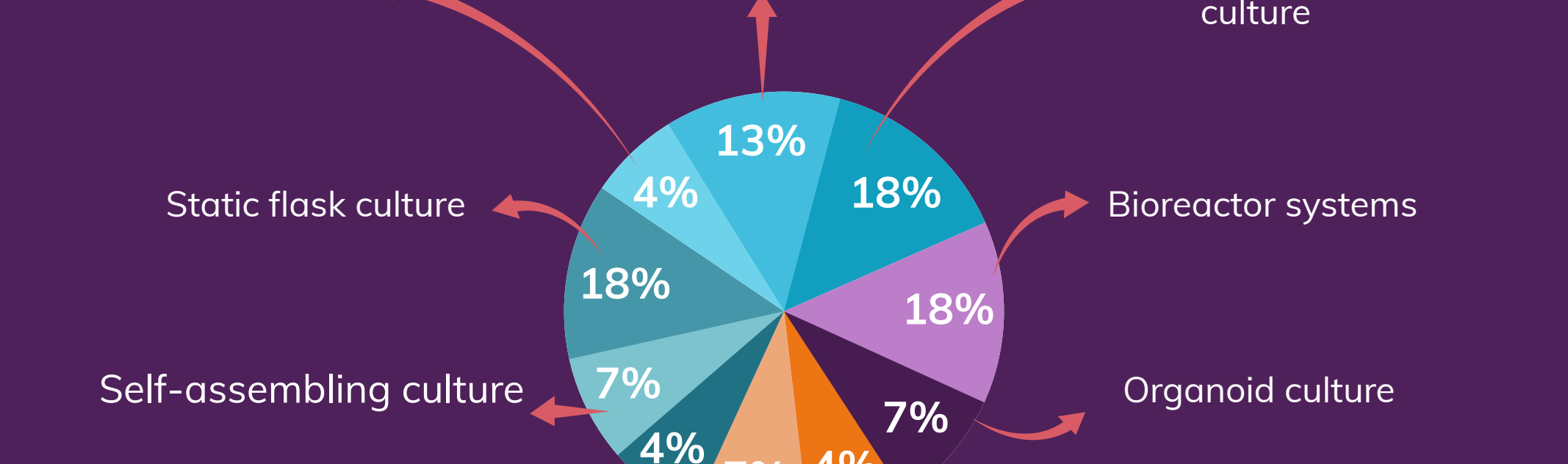
What cell type(s) do you most commonly work with?

- Embryonic stem cells 14%
- Induced pluripotent stem cells 18%
- Other 7%
- Osteoblasts 3%
- Mesenchymal stem cells 14%
- Hepatocytes 10%
- Adipose-derived stem cells 7%
- Myoblasts 3%
- Hematopoietic stem cells 7%
- Chondrocytes 3%
- Endothelial cells 7%
- Epithelial cells 7%

Where do you primarily source your cells from?

- Induced pluripotent stem cells (iPSCs) 3%
- Embryonic stem cells 7%
- Primary tissue biopsies 7%
- Cell banks 3%
- Hybrid sources (Combination of the above) 7%
- Commercial cell suppliers 23%
- Isolated from patient samples 23%
- Cell lines (e.g., established cell lines) 20%
- Animal sources 10%

What culturing methods or techniques do you use?



What criteria do you consider to be most important when selecting materials for scaffolds?

- 35% Biocompatibility
- 14% Mechanical properties
- 9% Degradation rate
- 9% Porosity and pore size
- 14% Biomimicry
- 19% Regulatory approval

What methods do you use for cell seeding onto scaffolds?

- Static seeding 56%
- Dynamic seeding 26%
- Rotary flask seeding 4%
- Perfusion seeding 4%
- Bioprinting 10%

What methods do you use to assess the biocompatibility of engineered tissues?

- 46% Cell viability assays
- 6% Histological analysis
- 16% Immunohisto-chemistry
- 23% Gene expression analysis
- 6% Inflammatory response assays
- 3% Biomechanical testing

How do you promote tissue maturation to achieve the desired functionality?

- 23% Biophysical stimulation
- 20% Coculture techniques
- 27% Chemical induction
- 7% 3D printing techniques
- 7% Perfusion bioreactors
- 16% Organoid culture systems

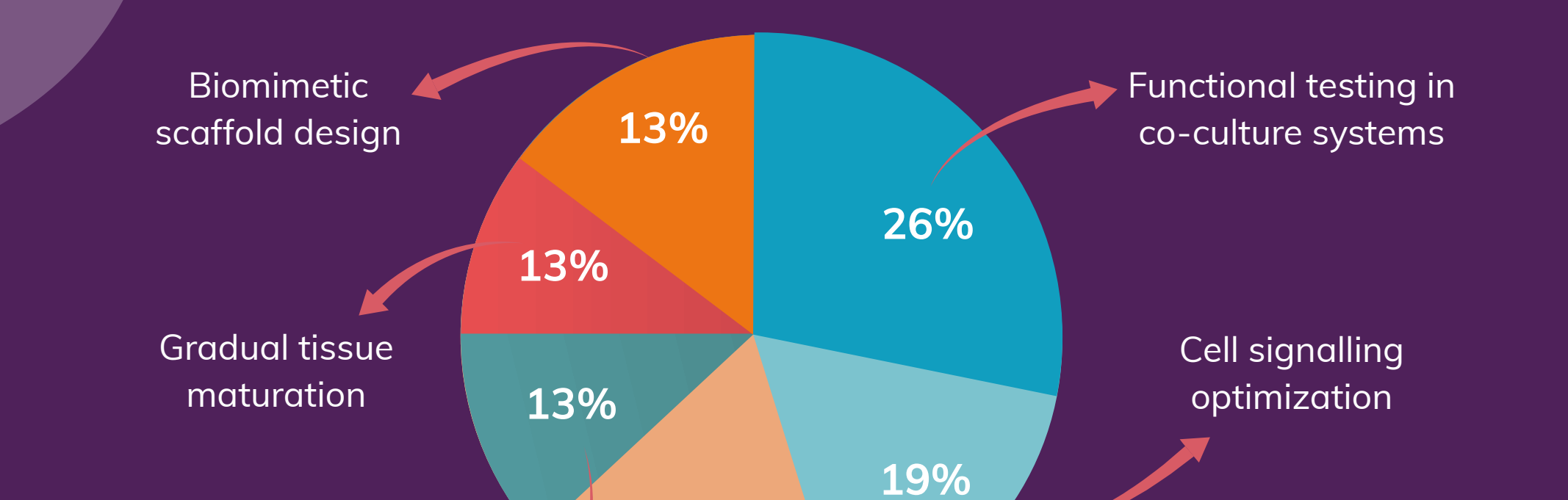
How do you address the challenge of vascularization?

- 45% Angiogenic growth factors
- 27% Coculture with endothelial cells
- 7% Incorporation of preformed vascular networks
- 7% Bioprinting blood vessels
- 7% Perfusion bioreactors
- 7% Microfabrication techniques

How do you ensure the long-term viability and stability of engineered tissues?

- 25% Biocompatible materials selection
- 7% Optimized scaffold degradation rates
- 46% Continuous monitoring and maintenance
- 7% Incorporation of cell-supportive matrices
- 15% Anti-inflammatory strategies

How do you ensure the functional integration of engineered tissues with surrounding native tissues in the later stages?



What do you foresee as the most promising developments or directions for the future of tissue engineering?

- 28% Advanced biomaterials and scaffolds
- 25% Innovation in vascularization techniques
- 25% Integration of bioprinting technologies
- 22% Artificial intelligence and computer modelling

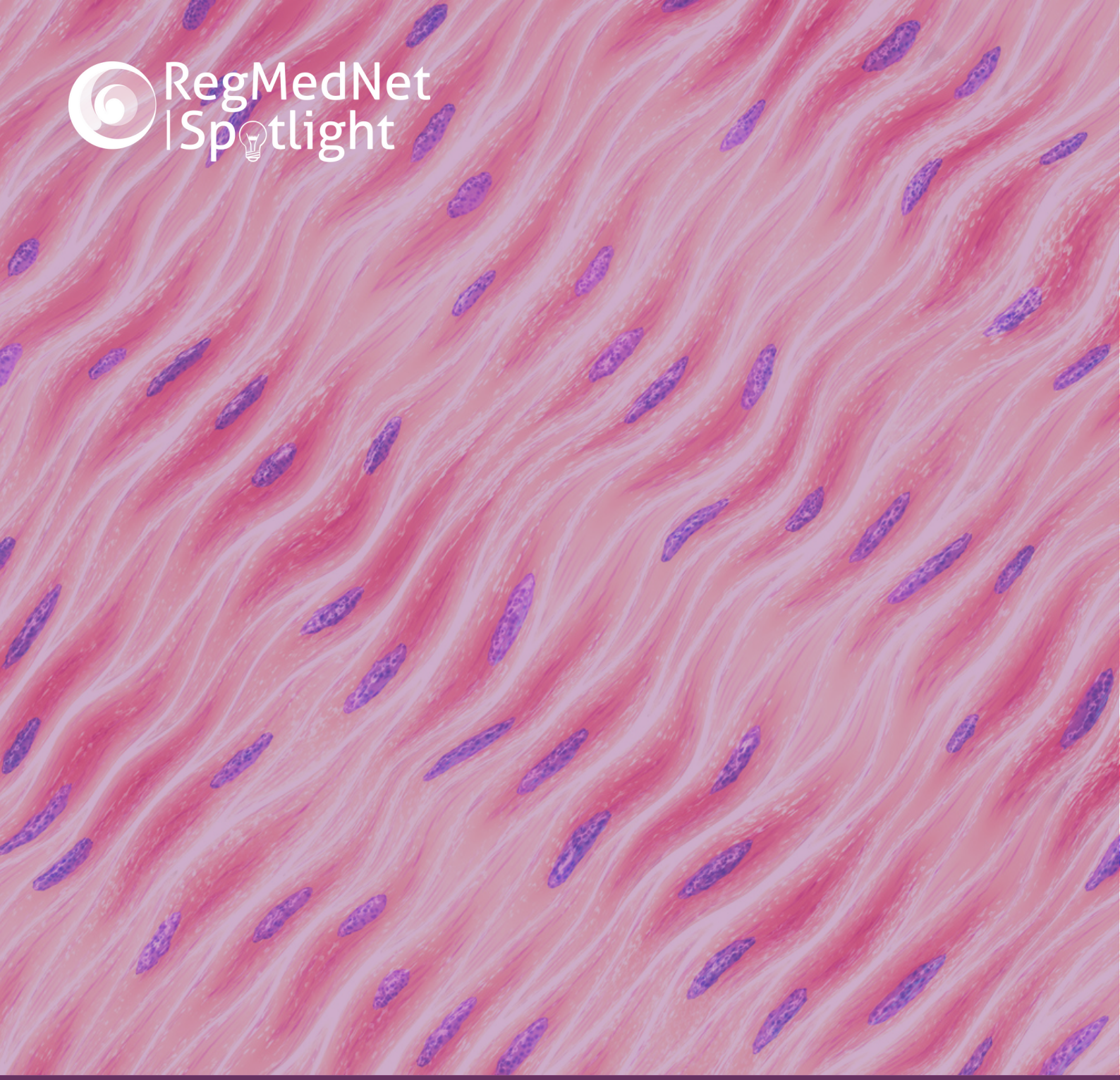
ABOUT THE RESPONDENTS

Location

- 18% North America
- 18% South America
- 39% Europe
- 14% Asia
- 4% Africa
- 7% Australia

Sector

- Academia 43%
- Government | NGO 4%
- CRO | CDMO 6%
- Hospital | Clinic 18%
- Pharma | Biotech 29%



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