Leveling Up Hydrogels

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Review

Leveling Up Hydrogels: Hybrid Systems in Tissue Engineering

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Hydrogels can mimic several features of the cell native microenvironment and have been widely used as synthetic extracellular matrices (ECMs) in tissue engineering and regenerative medicine (TERM). However, some applications have specifications that hydrogels cannot efficiently fulfill on their own. Incorporating reinforcing structures like fibrous scaffolds or particles into hydrogels, as hybrid systems, is a promising strategy to improve their functionality. We describe recent advances in the fabrication and application of these hybrid systems, where structural properties and stimuli responsiveness of hydrogels are enhanced while their ECM-like features are preserved. Furthermore, we discuss how these systems can contribute to the development of more complex tissue engineered structures in the rapidly evolving field of TERM.

Highlights

Native tissue mimicry is still a major challenge in TERM. Extracellular matrix (ECM) analogs should ideally provide biologically relevant biochemical/biophysical cues, in an adequate spatial-temporal manner, to properly stimulate cultured and/or recruited cells towards functional tissue formation.

Hydrogels are the most attractive ECM analogs, but it is difficult to tune them to mimic most of the native tissue-specific features alone without concomitantly hindering key biological processes. One promising strategy to overcome this relies on the use of hybrid systems, combining hydrogels with other structures.

Providing structural/mechanical reinforcement, spatial patterning/guidance, and/or stimuli responsiveness to hydrogel-based systems emerged as effective methodologies to more closely replicate the plethora of features and signals that native ECM presents to cells.

Native Extracellular Matrix Mimicry in Tissue Engineering

Cells are probably the first responders in wound-healing processes, which occur spontaneously whenever a tissue is injured [1]. Consequently, cell-based therapies have emerged as promising approaches towards tissue regeneration. However, due to poor control over cell delivery and retention at the injury site, major drawbacks have been observed [1]. This has turned attention to another key player in tissue engineering and regenerative medicine (TERM) [2] strategies: the extracellular matrix (ECM; see Glossary) [3].

ECM assemblies are highly dynamic, functioning both as a 3D support for cells and as active participants in controlling their behavior. Specificities of the different tissues result mainly from the dynamic biophysical and biochemical interactions between various cell types and their microenvironment [3]. In response to environmental stimuli, mature ECM can also undergo dynamic remodeling via reciprocate action between cells/ECM, allowing the tissue to maintain homeostasis and respond to stress [3,4]. ECM equilibrium can be disrupted, particularly when tissues are damaged. The human organism has a limited capacity to regenerate and repair damaged tissues depending, for example, on the damage extent. Bringing together the principles of life sciences and engineering [2] and knowing the basis of tissue morphogenesis and remodeling is essential for TERM strategies to succeed. Understanding how ECM components produced by cells assemble into macromolecules and then functional 3D structures is essential to perform a better selection of cells, **biomaterials** [5], and biomolecules [3,4].

Designed structures can provide an adequate microenvironment to guide cells in predictable ways to orchestrate tissue development and remodeling *in vivo* [3,4]. These may contain specific biophysical/biochemical cues and degradable components and may be used to carry/instruct cells and/or stimulate host cells. Thus, by using rationally designed 3D supports, bioactive components (e.g., diffusing/immobilized molecules, available in spatial and/or temporal gradients), and biophysical factors (architecture, stiffness) can be presented to cells. Whenever necessary, additional stimulation (e.g., hydrodynamic shear, mechanical strength, electrical signals) and dynamic culture can be provided by bioreactors [3,4]. Structures designed to mimic native ECMs allow cells to reside in a 3D environment where they are surrounded by other cells and/or matrix molecules. These can be derived from decellularized matrices, be synthetic/natural porous structures or hydrogels [3,4]. Yet, the main challenge for a proper mimic of the native cellular microenvironment relies on the fact that cell–ECM interactions are not yet fully understood, namely the complexity of their synergistic/antagonistic relationship and the spatiotemporal dynamics of tissue development and repair [6,7].

Theoretically, the ideal candidate TERM scaffold would be the natural ECM, which can be harvested from native tissues and subjected to decellularization processes [3]. The main advantage of decellularized

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ECM (dECM) as scaffolding material is that it supports and stimulates the formation of more specific tissue and less scar tissue [3]. Ideally, the decellularization process must remove all potentially immunogenic components, while preserving the original composition and structure of the native ECM as much as possible [3]. Ineffective decellularization is commonly associated with intense inflammatory responses, which can diminish or completely inhibit a proper remodeling [3,8]. Tissues from different donors decellularized by similar protocols can present significantly different dECM compositions after the process [3,9,10]. Despite all the advances in the field, the therapeutic use of dECM still faces challenges in terms of standardization and scaling-up, along with ethical and regulatory restrictions.

Drawbacks related to the use of dECM as scaffolding materials can be overcome by using artificial ECMs. These are generally based on 3D structures made of: (i) materials derived from naturally occurring molecules, or (ii) synthetic materials incorporating biomimetic features. When compared with the native ECM, artificial structures are simpler and easier to be industrially produced in large scales, possess lower batch-to-batch variation, face less regulatory issues, and are easier to manipulate (e.g., tailoring of mechanical and degradation properties) and process [4].

Over the past years, several types of ECM analogs have been explored. The intrinsic ability of hydrogels to more closely mimic several features of the native ECM made them emerge as favorite candidates (Box 1). Hydrogel biofunctionality has been largely improved through the modification of their network with cell-interactive cues. However, there are features that hydrogels cannot fully mimic. For instance, in applications where high strength and stiffness of the 3D supporting structure are required, increasing the polymer mass concentration and/or crosslinking density is a logical and straightforward strategy. However, when hydrogels are used for cell entrapment, the diffusion rate of cell metabolites, nutrients and bioactive factors is negatively impacted, hindering cell survival. Additionally, cells become more spatially confined, which affects their activity and interaction with the microenvironment, especially with presented ligands, and may thus compromise the regenerative potential of the system [11,12]. The combination of hydrogels with other types of structures can therefore improve their functionality.

This review focuses on studies that assess the *in vitro* and/or *in vivo* performance of hybrid structures, resulting from the combination of hydrogels with other structures (even if temporarily), and where the hydrogel component acts as the main 3D cell support. Studies where the hydrogel served as bioactive coating or filler, or where no *in vitro*/*in vivo* studies are presented, were not included.

Hydrogel-Based Hybrid Systems

The incorporation of different types of structures within a hydrogel matrix can significantly improve the overall features of a 3D system. In general, these can be created by integration of secondary polymer networks [creating hybrid networks, **interpenetrating networks (IPNs)** and semi-IPNs] [13] or by embedding, for example, micro- and nanoparticles or fiber-based 3D structures (obtained by, e.g., bioprinting or **electrospinning**) (Figure 1) [5,14]. The type of interaction of the reinforcing structures with the hydrogel can play a passive or active role, depending, for example, on whether those structures are simply physically embedded within the hydrogel network [15–17], or interact with it by means of covalent bonding [18]. More importantly, in cell-carrying systems, the type of hydrogel reinforcement will ultimately depend on the main biological outcome to be attained and can, therefore, be divided into three main categories: structural reinforcement, spatial patterning/guidance, and stimuli responsiveness (Box 2).

Tissue-Specific Applications

Biomimetic structures that substitute, support, and/or guide the regeneration of tissues have different requisites according to the characteristics of the native tissue. This section highlights some applications where hybrid 3D constructs have been used the most, namely in load-bearing and electroconductive tissue engineering, followed by a section dedicated to vascularization, due to its transversal relevance in TERM. Table 1 (Key Table) summarizes the details of the following and other studies.

Glossary

Angiogenesis: the process by which most vascular structures organize and are remodeled, from embryonic development onwards. New capillaries form via sprouting from pre-existing ones or vessels are split by the insertion of tissue pillars within pre-existing capillary networks.
Biomaterials: materials used to replace, restore, or regenerate a tissue or organ and its function as (part of) a medical device or advanced therapy medicinal products.
Carbon nanotubes (CNTs): class of nanomaterials consisting of a 2D hexagonal lattice of carbon atoms, bent and joined to form a hollow cylinder.
Electrospinning: material processing technology that combines polymer solutions or molten polymer with high electrical voltage to fabricate fine fibers. Fibers are deposited onto a collector with random or defined alignment.
Endochondral ossification (ECO): embryonic developmental pathway for long bone formation, it allows postnatal bone elongation and is the process by which most fractures heal. Here, chondrocytes within the developing limb bud undergo a coordinated sequence of proliferation and hypertrophy. This provides a growing template for bone formation.
Extracellular matrix (ECM): noncellular complex and dynamic collection of physiologically active molecules, including proteins and polysaccharides, secreted and arranged in a highly specialized organization by cells in a tissue.
Intramembranous ossification (IMO): process that leads to the formation of most of the bones that constitute the craniofacial skeleton; it is characterized by the direct differentiation of MSCs into osteoblasts that produce bone tissue.
Interpenetrating network (IPN): polymer network containing molecularly entangled chains of a second polymer but not covalently bonded to each other.
Vasculogenesis: *de novo* formation of capillary-like structures (not associated with pre-existing vascular structures) by endothelial

Box 1. Hydrogels as Artificial ECMs

Hydrogels are polymeric networks that can often be assembled under mild, cytocompatible conditions, which makes them ideal structures for cell entrapment and 3D culture. Additionally, they exhibit high water content and permeability, which facilitates the exchange of metabolites, gases, and nutrients with the extracellular milieu [4]. The compliant nature of hydrogels also allows embedded cells to be presented with adequate viscoelastic microenvironments, the properties of which can be tuned to match those of different types of native tissues.

In the earliest TERM applications, hydrogels were traditionally obtained by the covalent and/or physical cross-linking of polymer chains, being essentially used as passive/inert 3D supports for embedded cells [4,55]. Without proper bioactive cues and biophysical properties, the interactions of embedded cells with the surrounding environment was compromised. This negatively affected cellular activity, endogenous extracellular matrix (ECM) deposition, and proper neo-tissue maturation [4,55]. However, the hydrogel research field has been rapidly evolving towards the design of materials able to promote dynamic interactions between cells and their surroundings, in a controlled way. The properties and complexity of the native ECM, like bioadhesiveness and proteolytic susceptibility, along with many other biochemical/biophysical cues, and associated spatial-temporal dynamics, are being progressively better mimicked.

Therefore, supported by the increasing knowledge on cell–ECM interactions, hydrogels with optimized properties have been developed to foster new tissue formation and maturation. For example, it is now well established that the fate commitment of mesenchymal stem/stromal cells populations correlates with the physical properties of 3D microenvironments, such as stiffness and stress relaxation [56]. By using 3D synthetic matrices with tunable stress relaxation rate (independently of the hydrogel initial elastic modulus, degradation, and cell-adhesion ligand density), Chaudhuri and colleagues [56] observed that spreading, proliferation, and osteogenic differentiation of MSCs are all enhanced in cells cultured in hydrogels with faster relaxation. Another 3D ECM network property, internal topography, also influences cellular behavior. By positioning protein-coated magnetic beads into specific spatial configurations, the topography of 3D matrices can be internally controlled [57] and, for example, guide the dendritic protrusions of embedded cells, independently from the type/stiffness or material. As scaffolds, hydrogels should also ideally degrade at the rate that new tissue forms and this can occur either by chemical degradation (typically by hydrolysis of polymer chains) or by cell-driven (enzymatic) degradation, taking advantage of pathophysiological mechanisms. These mechanisms can be explored not only to modulate degradation and favor new tissue formation, but also to promote delivery of bioactive molecules. For example, a matrix metalloproteinase-sensitive hydrogel can be enzymatically degraded to allow new tissue formation and host cellular infiltration, while locally delivering/presenting specific biomolecules [58].

precursor cells or angioblasts. It takes place during early embryonic development and can also occur in adults, namely to re-vascularize a tissue after extensive damage.

Bone

During skeletogenesis, bone formation occurs by two distinct means: **intramembranous ossification (IMO)** and **endochondral ossification (ECO)** [19], with IMO being the most replicated process in the TERM field. Native bone ECM can be generally described as a composite matrix of collagen (organic phase) reinforced with calcium phosphate nanocrystals (mineral phase), arranged in a highly organized structure [19].

One of the main goals of reinforcing hydrogels in bone TERM is to obtain mechanically improved structures, while allowing cellular activities such as proliferation or ECM secretion to occur within a compliant hydrogel. For example, Heo and colleagues [20] incorporated gold nanoparticles within gelatin-based hydrogels and further reinforced these with fibrous polylactide scaffolds. The compressive modulus of the hydrogel alone could be significantly increased by the scaffolds, presenting similar values to human mandibular bone. Hybrid structures promoted significantly higher gene expression of osteogenic-specific factors of entrapped human adipose-derived stem cells.

As in other vascularized tissues, tissue engineered (TE) structures used in bone TERM strategies often fail to properly engraft after implanted, mainly due to avascular necrosis, occurring especially at the construct's core [19,21]. 3D fibrous scaffolds can be used to reinforce the hydrogel component and, if rationally designed, these may also improve the nutrient and oxygen diffusion into the structures. By incorporating microchannels into the constructs, Kang and coworkers [15] overcame the diffusion limit of 100–200 μm for cell survival in engineered tissues. The 3D structures were fabricated using

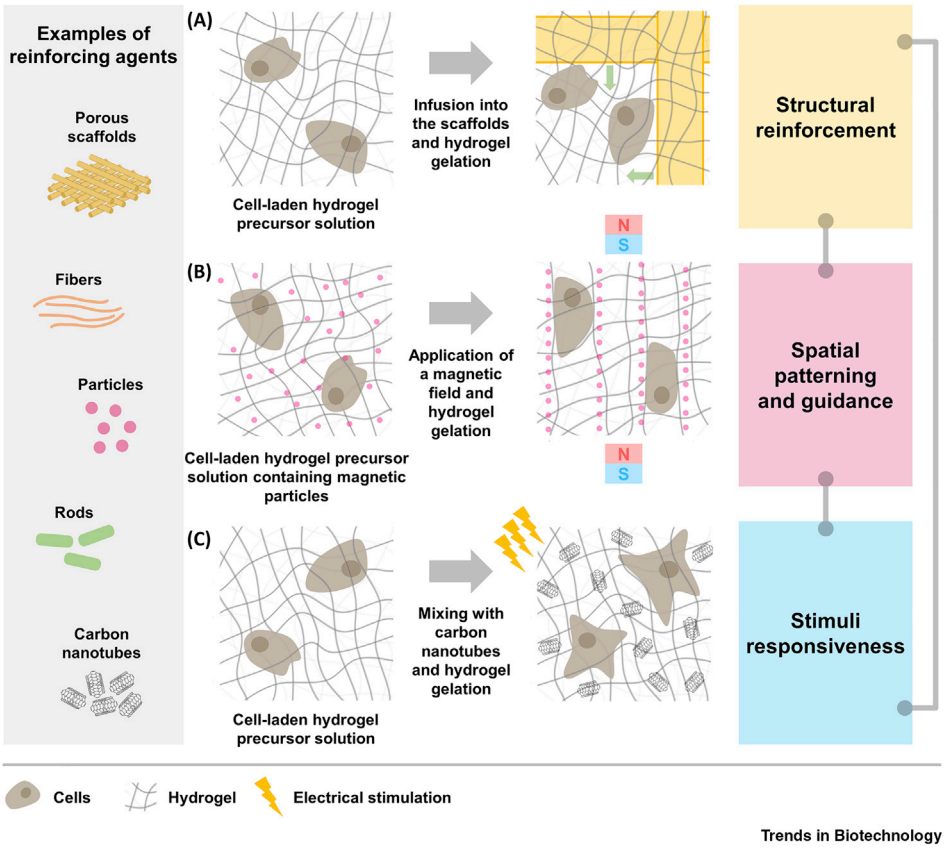

Figure 1. Schematics of the Different Types of Hydrogel Reinforcing Agents and Examples of the Resultant Properties.

(A) The incorporation of porous scaffolds within cell-laden hydrogels can result in the increase of the hybrid construct stiffness [16,18,78]. At the microscopic level, the generated stiffness gradients propagating from the scaffold fibers through the hydrogel component (green arrows) may also influence the cellular behavior [67]. (B) By dispersing magnetic particles (MPs) within a hydrogel precursor solution, the internal 3D patterning of hydrogels can be tuned by applying a magnetic field and control the orientation of the MPs. This consequently leads to a specific hydrogel mesh internal organization during gelation, which can influence cellular spatial orientation [44,57]. (C) The responsiveness of hydrogels to external stimuli, like electrical potential, can be tuned by incorporating carbon nanotubes, for example, within the matrix. This can improve the response of embedded cells, the activity of which is electrosensitive [42,62].

a combination of poly(ε-caprolactone) (PCL)/tricalcium phosphate and cell-laden filaments composed of human stem cells in a gelatin/fibrinogen/hyaluronic acid/glycerol hydrogel.

As an alternative to the use of IMO in bone tissue regeneration strategies, ECO mimicry has been proposed [22]. In a recent *in vivo* study, Thompson and colleagues [23] observed that scaffolds with embedded chondrogenically primed mesenchymal stem/stromal cells (MSCs) promoted enhanced repair of critical-sized bone defects, as compared with osteogenically induced cells. In this context, structures combining ECO with hybrid scaffolds may also improve the outcome of bone tissue regeneration, as suggested by the work of Daly and colleagues [16] (Figure 2A). In this study, bioprinting was used to engineer an anatomically accurate, mechanically reinforced, hypertrophic cartilage hybrid structure. PCL- and MSC-laden hydrogel filaments were co-deposited in a layer-by-layer fashion. The reinforcement of the hydrogel with PCL fibers increased the construct compressive modulus around 350-fold, providing the necessary stiffness to implant the immature cartilaginous

Box 2. Types of Hydrogel Reinforcements According to Expected Main Biological Effect

Structural Reinforcement

Several works reported enhanced mechanical properties, and hence biofunctionality, of hydrogel-based systems incorporating different types of structures, including fibers [15,16,18,27,28,52,59–61], particles [62–65], or by using interpenetrating/semi-interpenetrating networks [13]. In this specific case of structural reinforcement, the way that reinforcing structures interact with the network dictates the way that stress propagates within the structure. Cellular activity is largely influenced by the mechanical properties of the 3D microenvironment [66] and, even if not in direct contact with cells, the reinforcing structures can impact the cellular behavior by the referred stress propagation [67]. The structural reinforcement of the hybrid constructs tends to have a more protective and supportive role of the hydrogel component, as shown by Kang [15] or Daly [16] and corresponding coworkers. Reinforcing agents in the nanoscale may have a more local and direct impact on the hydrogel network and cellular microenvironment [14]. Nanomaterials can be integrated within the hydrogel network and improve their mechanical properties via covalent or noncovalent interactions [17]. Additionally, the high aspect ratio presented by the majority of nanomaterials allows the use of relatively low volume fraction of these to obtain high fold increases on hydrogel viscoelastic properties [68].

Spatial Patterning and Guidance

Cells spatially organize in specific patterns and orientations within the majority of tissues and several functions heavily rely on cell shape and polarity, which have implications in directional migration, basal–apical polarity, and asymmetrical cell division [69]. These processes are intimately related to tissue homeostasis [66] and have been studied to improve the design of synthetic extracellular matrices (ECMs) [70,71]. The internal topography of hydrogels can be tuned via different methodologies such as spatial positioning of bioactive cues, selective removal/reshaping of material from a bulk gel [72,73], or by using reinforcing agents [44–46,57]. For instance, McMurtrey [46] assessed the behavior of neural cells embedded within hydrogels integrating aligned nanofibers. A significant increase on cell alignment, in parallel to the nanofibers, and on the distance over which neurites could extend was observed within the 3D hybrid structures when compared with controls.

Stimuli Responsiveness

Hydrogels can be tuned to respond to different stimuli in a specific manner, similarly to the native ECM. Hydrogel responsiveness may be achieved via incorporation of structures with electrical, optical, or thermal conductivity, for example, which are not intrinsic properties of commonly used polymers [42,62,74,75]. This may enhance the outcomes of electroactive tissues engineering like muscle and nerve, for example, where the native ECMs and cells are responsive to such stimuli [76,77]. Shin and colleagues [62] developed gelatin-based hydrogels reinforced with carbon nanotubes (CNTs). This improved both mechanical and electrophysiological properties of the hydrogels due to the networks formed by CNTs. Cardiomyocytes seeded on the hybrid hydrogels showed three times higher spontaneous synchronous beating rates compared with those cultured on pristine hydrogels.

structures into load-bearing locations. Moreover, endochondral bone formation was observed *in vivo* after 12 weeks of subcutaneous implantation.

Cartilage

Cartilage is composed by one cell type only, the chondrocytes, which are embedded within a highly permeable ECM, mainly consisting of proteoglycans and collagen type II [24]. Amongst the three types of cartilage in the body, the TERM field has mainly focused on articular cartilage (AC), since most cartilaginous defects result from traumatic injuries or degenerative joint diseases [24]. Although cartilage appears to be a rather simple aneural, avascular, and alymphatic tissue, its complexity lies with its composition and architecture. During AC development, spatiotemporal gradients of chondrogenic signaling factors drive MSCs to condense in structurally different zones [24], namely: superficial zone (lubrication); middle/deep zone (compressive strength, resistance to deformation); and calcified zone (load transmission to underlying bony tissue). These zones have a specific combination of biochemical, biophysical, and cellular factors [24,25]. Thus, a synthetic ECM analog for AC should ideally be able to mimic this zonal organization.

Key Table

Table 1. Application of Hydrogel-Based 3D Hybrid Structures in Tissue Engineering and Regenerative Medicine[a]

Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
Bone	Alginate	α-TCP	MC3T3-E1 preosteoblast cell line	–	The mechanical properties of the 3D printed scaffolds of alginate filaments were significantly enhanced by the integration of the α-TCP. Cells embedded within the alginate hydrogel shell maintained viability for up to 35 days.	[79]
	Alginate (RGD-modified, γ-irradiated)	PCL scaffolds (FDM)	BM-MSCs (porcine)	Subcutaneous implantation (Balb/c nude mice)	The scaffold reinforced hydrogels resulted in a 350-fold increase in the constructs compressive modulus when compared with the hydrogels alone. The cell-laden hybrid constructs supported the development of vascularized bone containing trabecular-like endochondral bone with a supporting marrow structure.	[16]
	Chitin and PBSC	Fibrin nanoparticles; magnesium-doped bioglass	ASCs (rabbit); UVECs (human); (*ex vivo*: mouse aortic ring angiogenesis sprouting assay)	–	Hydrogels containing magnesium-doped bioglass showed early initiation of differentiation and higher expression of alkaline phosphatase and osteocalcin. The hybrid system showed enhanced sprouting in an *ex vivo* mouse aortic ring angiogenesis assay.	[80]
	Mixture of gelatin, fibrinogen, HyA, and glycerol	PCL scaffolds doped with TCP nanoparticles (FDM) and pluronic F-127 (3D extrusion printing)	AFSCs (human)	Calvarial bone defect model (Sprague Dawley rats)	The hybrid structures fabricated by 3D extrusion printing showed newly formed vascularized bone tissue. The untreated defect and scaffold-only treated control groups showed fibrotic tissue ingrowth and minimal bone tissue formation restricted to the periphery of the implant.	[15]
	GelMA	Nanosilicates (Laponite®)	BM-MSCs (human)	Subcutaneous implantation in immunocompetent rats (Wistar rats)	BM-MSCs embedded within the 3D hybrid hydrogels were able to differentiate into the osteogenic lineage without the addition of growth factors. *In vivo* biocompatibility and minimal inflammatory response were demonstrated.	[81]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
	GelMAte	Nanosilicates (Laponite®)	MC3T3-E1 preosteoblast cell line	–	The presence of nanosilicates increased the hydrogel compressive modulus and promoted osteogenesis in the absence of osteoinductive factors.	[82]
	GelMAde	Nanodiamonds	ASCs (human)	–	The nanodiamonds incorporated within the hydrogel increased the network stiffness.	[83]
	GelMAte	PLA scaffolds (FDM); RGD-modified gold nanoparticles	ASCs (human)	–	The incorporated nanoparticles increased the stiffness of the hydrogels; incorporation of RGD-modified nanoparticles within the hydrogels promoted significantly higher gene expression of osteogenic specific factors by human ASCs.	[20]
	GelMAte	PLA	BM-MSCs (human); UVECs (human)	–	A highly osteogenic construct with organized vascular networks was generated using the hybrid system. The dynamic biochemical environment provided a controlled and continuous stimulus for vascularized bone regeneration. It accelerated endothelial cells and osteoprogenitors towards a more rapid formation of vessel networks.	[84]
	Gellan gum	Hemicellulose microfibers	ASCs (rat)	Medication-related osteonecrosis of the jaw model (Sprague Dawley rats)	The incorporation of microfibers into the hydrogels could promote increased mechanical strength. The spreading, viability, and proliferation of ASCs within hybrid structures were enhanced and supported the osteogenic differentiation and bone regeneration *in vivo*.	[85]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
Cartilage	Mixture of gelatin, fibrinogen, HyA, and glycerol	PCL scaffolds (FDM) and pluronic F-127 (3D extrusion printing)	Ear chondrocytes (New Zealand white rabbits)	Subcutaneous implantation (athymic nude mice)	The hybrid structures fabricated by 3D extrusion printing could generate complex, human ear-shaped tissue construct containing cartilage tissue possessing histological and mechanical characteristics of human auricles after implantation *in vivo*.	[15]
	COL I and COL I-genipin	Cadmium selenide quantum dots	BM-MSCs	Subcutaneous implantation (nude mice) and cartilage-only patellar defect (Sprague Dawley rats)	The introduction of the quantum dots considerably strengthened the stiffness of the collagen hydrogels. The hybrid structures promoted the proliferation of BM-MSCs, induced cartilage-specific gene expression, and increased secretion of glycosaminoglycan.	[65]
	PLGA-PEG-PLGA copolymer	PCL scaffolds (FDM)	BM-MSCs	–	MSCs were able to chondrogenically differentiate within the hybrid scaffolds with a greater amount of cartilage-specific matrix production compared with the PCL scaffold or gel.	[86]
	GelMAde	PA/PCL, PA/PLA, or PA/PLGA amphiphilic macromonomers (3D printed scaffolds)	Chondrocytes (equine)	–	The hybrid construct stiffness and degradation kinetics could be tuned. Cells viability remained largely unaffected by the printing process.	[87]
	GelMAde	Methacrylated PHMGCL and PCL (scaffolds by FDM)	Chondrocytes (human)	Subcutaneous implantation (athymic rats)	The grafting of the hydrogels to the fibrous scaffolds resulted in improved interface-binding strength between the hydrogel and the thermoplastic polymer and resistance to repeated axial and rotational forces. Chondrocytes embedded within the 3D hybrid constructs were able to form cartilage-specific matrix both *in vitro* and *in vivo*.	[18]
	GelMAde and HyA	PCL (scaffolds by melt-electrospinning writing)	Chondrocytes (human)	–	The stiffness of the 3D hybrid structures increased synergistically (up to 54-fold) compared with hydrogels or microfiber scaffolds alone. The stiffness and elasticity	[27]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
					of the composites approached that of articular cartilage tissue. The chondrocytes embedded within the hybrid system were viable, retained their round morphology, and responded to an *in vitro* physiological loading regime in terms of gene expression and matrix production.	
	GelMAde	PCL (scaffolds by melt-electrospinning writing)	Chondrocytes (equine)	–	The constructs were able to mimic functional properties of both the superficial tangential and middle/deep zones of native cartilage. The composite structures were able to support neo-cartilage formation upon physiologically relevant mechanical stimulation.	[29]
	Silk	Silk microfibers	Chondrocytes (bovine)	–	The fiber reinforcement resulted in more mechanically robust constructs after 42 days in culture compared with silk hydrogels alone.	[88]
	SPELA	PLA (electrospun fibers)	BM-MSCs	–	The hybrid structure mimicked zone-specific characteristics of articular cartilage, using nanofiber spatial orientation and growth factors to create distinct zones within the hydrogel. This influenced the chondrogenic differentiation of the MSCs and their behavior throughout the 3D construct.	[28]
	SPELA	PLA (electrospun fibers) and HAp nanoparticles	BM-MSCs (human)	–	The zone-specific characteristics of articular cartilage were mimicked by using stiffness gradients and growth factors to create distinct zones within the SPELA hydrogel. This influenced the chondrogenic differentiation of the MSCs and their behavior throughout the 3D construct.	[89]
Osteochondral	Agarose	HAp particles	Chondrocytes (bovine)	–	Higher matrix deposition and mineralization was observed when HAp was added to the hydrogel. Higher matrix content translated	[90]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
					into significant increases in both compressive and shear mechanical properties.	
	Alginate	HAp and TCP fibrous scaffolds (projection-based microstereolithography)	Chondrocytes (New Zealand white rabbits)	Full-thickness osteochondral defect (New Zealand white rabbits)	In the 3D hybrid scaffolds group, the newly generated cartilage tissues were morphologically similar to native ones and connected to the surrounding tissues. In the empty defect and hydrogel-only scaffold groups, the defects were filled with fibrous tissues.	[91]
	Atelocollagen; supramolecular HyA (CB[6]/DHA-HyA)	PCL scaffolds (FDM)	TSCs (human)	Full-thickness osteochondral defect (New Zealand white rabbits)	The multilayered hybrid scaffold accelerated the regeneration of the osteochondral defect, with corresponding different tissue formation.	[59]
Skeletal muscle	Mixture of gelatin, fibrinogen, HyA, and glycerol	Pluronic F-127 and PCL (3D extrusion printing)	C2C12 myoblast cell line	Subcutaneous implantation (nude rats)	The printed cells stretched along the longitudinal axis of the constructs with high cell viability. Cells within the 3D hybrid system without the PCL support did not show cellular alignment. Muscle-like structures with aligned myotubes were observed on the 3D hybrid construct. The retrieved muscle constructs presented well-organized muscle fiber structures, nerve (neurofilament) contacts, and vascularization. The electromyographies revealed that the compound muscle action potential of the TE constructs was of 3.6 mV, compared with 10.7 mV for the control gastrocnemius muscle, and 0 mV for the negative controls.	[15]
	GelMAte	CNTs	C2C12 myoblast cell line	–	Hydrogels with aligned CNTs showed anisotropic electrical conductivity and superior mechanical properties compared with pristine hydrogels or hydrogels containing randomly distributed CNTs. Skeletal muscle cells yielded a higher number of functional myofibers on the hydrogels with aligned CNTs.	[34]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
	PEGS-M	PCL/SF/PANI (network of dry-wet electrospun nanofiber yarns)	C2C12 myoblast cell line	–	The core-shell scaffolds guided the myoblast alignment and differentiation and the hydrogel shell that provided a suitable 3D environment for nutrition exchange and mechanical protection.	[92]
Cardiac	COL I	CNTs	Cardiomyocytes (neonatal, rat); LX-2 hepatic cell line	–	Improved mechanical strength and electrical performance of COL I hydrogels, along with increased rhythmic contraction area by the cells.	[37]
	GelMAte	Gold nanorods	Cardiomyocytes (neonatal, rat)	–	The gold nanorods promoted electrical conductivity and increased mechanical stiffness of the hydrogels. The hybrid scaffolds supported synchronous tissue-level beating.	[17]
	GelMAte	PCL/SF/CNTs (network of electrospun nanofiber yarns)	Cardiomyocytes (neonatal, rat); endothelial cells	–	The 3D hybrid structure supported cellular orientation and maturation and mimicked cardiac tissue anisotropy by having cardiomyocytes seeded on the nanofibers yarns and endothelial cells embedded within the hydrogel.	[38]
Nerve	Agarose and methylcellulose	PLLA and fibronectin (electrospun fibers)	–	Implantation in the striatum (Wistar rats)	The infiltrating macrophages/microglia and resident astrocytes from the brain tissue were able to locate the fibers and the cues for migration into the hybrid matrix.	[93]
	Collagen	CNTs	MSCs	–	The incorporation of CNTs within the hydrogel significantly stimulated neural markers and secretion of neurotrophic factors.	[41]
	Collagen	MPs	PC12 cell line; neurons from the CNS of adult medicinal leeches	–	Neurons within the 3D magnetically induced gels exhibited normal electrical activity and viability. They presented an elongated co-oriented morphology, relying on the particle strings and fibers as supportive cues.	[44]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
	COL I and Matrigel®	CNTs	Neonatal DRGs from Sprague Dawley rats	–	Neurite outgrowth increased 3.3-fold in the CNT-hydrogels when compared with the hydrogels alone. The electrical stimulation resulted in a 7.0-fold increase in outgrowth relative to the unstimulated CNT-free hydrogel.	[42]
	HyA and Matrigel®	PCL (electrospun fibers)	SH-SY5Y cell line	–	The incorporation of the aligned nanofibers within the hydrogels improved the alignment of neurites, enabled significant neurite tracking of nanofibers, and increased the distance over which neurites could extend.	[46]
	OPF	GO acrylate; CNT-PEG acrylate	PC12 cell line	–	Neurite development of PC12 cells was observed to be largely stimulated on the composite hydrogel compared with the neutral OPF hydrogel.	[94]
	PEG	MPs incorporated within rod-shaped microgels	DRG (chicken); L929 fibroblasts cell line	–	The generated unidirectional orientation of the rods incorporating nanoparticles within the hydrogels was strongly sensed by the cells resulting in parallel nerve extension.	[45]
Vascular	COL I	PLGA (electrospun fibers)	UVECs (human)	Subcutaneous implantation in immunodeficient nude mice	PLGA electrospun fibers guided the formation of vascular-like structures within the collagen gels. The integrity of their lumen and structure was retained after the PLGA fibers resorption.	[95]
	Fibrin	PPF scaffolds (3D printing)	BM-MSCs; UVECs (human)	Subcutaneous implantation in mice with severe combined immunodeficiency (SCID C.B17)	*In vitro* prevascularization supports *in vivo* vascularization in PPF/fibrin scaffolds.	[96]
	HyA	PCL (electrospun fibers)	ASCs (human); BM-M	Subcutaneous implantation (dorsum) of Lewis rats and soft tissue defect model in	The HyA hydrogel reinforced with PCL electrospun nanofibers was able to generate tubular endothelial structures with	[52]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
				New Zealand white rabbits	lumen and branching. Such organization was not observed within hydrogels alone with similar mechanical properties. The presence of the microfibers was shown to promote improved biological outcomes, by providing spatial guidance to cells towards vasculogenesis within hydrogels.	
	PEG, fibrin, Matrigel®, alginate, and agarose	Carbohydrate glass (mixture of glucose, sucrose, and dextran) fibrous scaffolds (FDM)	UVECs (human)	–	The printed rigid 3D filament networks of carbohydrate glass were used as cytocompatible sacrificial templates to generate cylindrical networks within different hydrogels. The 3D networks were lined with endothelial cells that were embedded within the hydrogels and perfused with blood under high-pressure pulsatile flow. The perfused vascular channels sustained the metabolic function of primary rat hepatocytes in engineered tissue constructs.	[53]
	Gelatin	Pluronic F-127 (3D extrusion printing)	MSCs (human); UVECs (human); NDFs (human)	–	A perfusable thick construct was created by using a 3D printed silicon chip, a Pluronic F-127 network as cytocompatible sacrificial template, and a cell-laden hydrogel of gelatin to generate thick human tissues (>1 cm). The perfusion system could be used for the delivery of cells to endothelize the whole construct and to distribute the growth factors to promote differentiation of human MSCs embedded within the hydrogel.	[54]
Skin	Atelocollagen (type I, porcine)	Silver nanoparticles	–	Full-thickness burns (Sprague Dawley rats)	The collagen hydrogels modified with histidine and reinforced with silver nanoparticles demonstrated increased mechanical strength, better biocompatibility, antibacterial properties, and accelerated wound closure.	[97]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
	COL I	TIGR® surgical mesh (knitted mesh of two copolymers: TMC/PLA and TMC/PLGA); PLGA (electrospun mesh)	–	Full-thickness skin defects (immuno-incompetent rats)	The hybrid 3D skin substitutes homogeneously developed into a well-stratified epidermis over the entire surface of the grafts. The dermal component of the grafts was well vascularized.	[98]
	Gelatin and COL I	PCL scaffolds (FDM)	Dermal fibroblasts (human); epidermal keratinocytes (human)	–	The hybrid 3D construct prevented the contraction of the gels during tissue maturation due to the integrated PCL. This skin model revealed favorable biological characteristics, including a stabilized fibroblast-stretched dermis and stratified epidermis layers after 14 days.	[99]
Heart valve	GelMAte and HyAMA	PAN (network of electrospun nanofiber yarns)	Aortic valve interstitial cells (normal and diseased; human)	–	Hybrid scaffolds prevented matrix shrinkage and maintained physiological fibroblastic phenotype in both normal and diseased aortic valve interstitial cells. When compared with hydrogels or nanofiber yarns alone, the hybrid scaffolds restrained the pathological differentiation of diseased aortic valve interstitial cells into myofibroblasts and osteoblasts.	[100]
	GelMAte and HyAMA	PGS/PCL (electrospun fibers)	Mitral valve interstitial cells (sheep)	–	Cell viability and metabolic activity were similar amongst all scaffold types. The presence of the hydrogel improved the spatial distribution of mitral valve interstitial cells within the hybrid constructs.	[101]
Urethra	Fibrin	PCL/PLCL scaffolds	Bladder urothelial cells (rabbit); smooth muscle cells	–	The tubular hybrid scaffolds could mimic a natural urethral base-membrane and facilitate contacts between the hydrogel-embedded cell types on both sides of the scaffold.	[102]
Tendon/ Ligament	GelMAte	PCL and GelMAte (electrospun fibers)	ASCs (human)	–	Photocrosslinking of sheets of hybrid scaffolds allowed formation of multilayered constructs that mimic the structure of native tendon tissues. Cells within the constructs remained responsive to topographical cues and tenogenic factors.	[103]

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Tissue or biological structure	Hydrogel	Reinforcing structure	*In vitro*	*In vivo*	Main readouts	Refs
Endocrine	Alginate	PCL scaffolds (FDM)	Islets of Langerhans (human); *in ovo*: chicken chorioallantoic membrane (CAM) assay	–	The islets encapsulated within the alginate core surrounded by the VEGF-functionalized PCL scaffold showed functional response to glucose stimuli comparable with free-floating islets. Thus, the platform showed potential to support rapid vascularization and islet endocrine function re-establishment.	[104]

[a]Abbreviations: AFSCs, amniotic fluid-derived stem cells; ASCs, adipose-derived stem cells; BM-M, bone marrow-derived macrophages; BM-MSCs, bone marrow-derived mesenchymal stem cells; CB[6], cucurbit[6]uril; CNTs, carbon nanotubes; COL I, collagent type I; DAH, 1,6-diamoniohexane; DRG, dorsal root ganglia; FDM, fused deposition modeling; GelMA, gelatin methacryloyl; GelMAde, gelatin methacrylamide; GelMAte, gelatin methacrylate; GO, graphene oxide; HAp, hydroxyapatite; HyA, hyaluronic acid; HyAMA, methacrylated hyaluronic acid; MPs, magnetic particles; MSCs, mesenchymal stem cells; NDFs, neonatal dermal fibroblasts; PA, poloxamer 407; PAN, polyacrylonitrile; PANI, polyaniline; PBSC, poly(butylene succinate); PCL, poly(ε-caprolactone); PEG, poly(-ethylene glycol); PGS, poly(glycerol sebacate); PEGS-M, methacrylated poly(ethylene glycol)-co-poly(glycerol sebacate); PLA, polylactide; PLCL, poly(lactide-co-caprolactone); PLGA, poly(D,L-lactide-*co*-glycolide); PLLA, poly(L-lactide); PHMGCL, poly(hydroxymethylglycolide-co-ε-caprolactone); PPF, poly(propylene fumarate); RGD, arginine-glycine-aspartic acid; OPF, oligo(poly(ethylene glycol) fumarate); SF, silk fibroin; SPELA, star acrylate-terminated lactide-chain-extended polyethylene glycol macromer; TCP, tricalcium phosphate; TE, tissue engineered; TMC, trimethylene carbonate; TSCs, turbinate-derived mesenchymal stem cells; UVECs, umbilical vein endothelial cells; VEGF, vascular endothelial growth factor.

Most of the studies using cartilage ECM analogs have been performed with hydrogels. However, in order to match the viscoelastic properties of AC, attempts to reach sufficiently high hydrogel stiffness come at the cost of other biological functions. For example, this can negatively influence the outcome of chondromimetic structures by inducing hypertrophic differentiation of entrapped chondrogenically induced MSCs [26]. Interestingly, reinforcing hydrogels with other structures can increase the overall stiffness of the construct, while keeping the hydrogel component with adequate compliance and permeability [27–30]. Visser and colleagues [27] assessed the effect of fiber-reinforced hydrogels in the production of cartilaginous tissue by chondrocytes. Highly porous PCL (93% porosity) scaffolds were produced using direct-writing melt-electrospinning to reinforce gelatin-based/hyaluronic acid hydrogels. The stiffness of the hybrid structures approached that of AC, while maintaining physiologically relevant elasticity. Furthermore, gene expression data suggested upregulation of matrix mRNA expression by chondrocytes within the hybrid structures. In a more recent study [29], the same polymers and techniques were used to obtain improved structures, increasing mechanical integrity after implantation and inducing guided zonal tissue formation. Bilayered constructs consisting of a densely distributed crossed fiber mat (superficial tangential zone) and a uniform box structure (middle and deep zone) presented a stress relaxation response comparable with native cartilage tissue. Furthermore, chondrocytes embedded within these hybrid constructs produced similar levels of cartilage-specific ECM components when cultured under mechanical conditioning without specific growth factor supplementation versus the static conditions with supplementation.

Hybrid structures can also be of great help towards proper replication of AC stratified structure and independent effect of chemical and physical factors on zone-specific cellular activity. For example, the Moeinzadeh team [28] (Figure 2B) was able to assess the specific effect of: (i) a physical cue (addition/orientation of incorporated nanofibers); (ii) a mechanical cue (zone-specific matrix modulus); and (iii) two biomolecular factors (transforming growth factor β1 and a zone specific growth factor) on the chondrogenic differentiation of human MSCs. A synthetic polymer-based hydrogel was reinforced with polylactide electrospun nanofibers. The hybrid structures presented tunable modulus and degradation time and were able to mimic the zonal differences on AC tissue. On the superficial

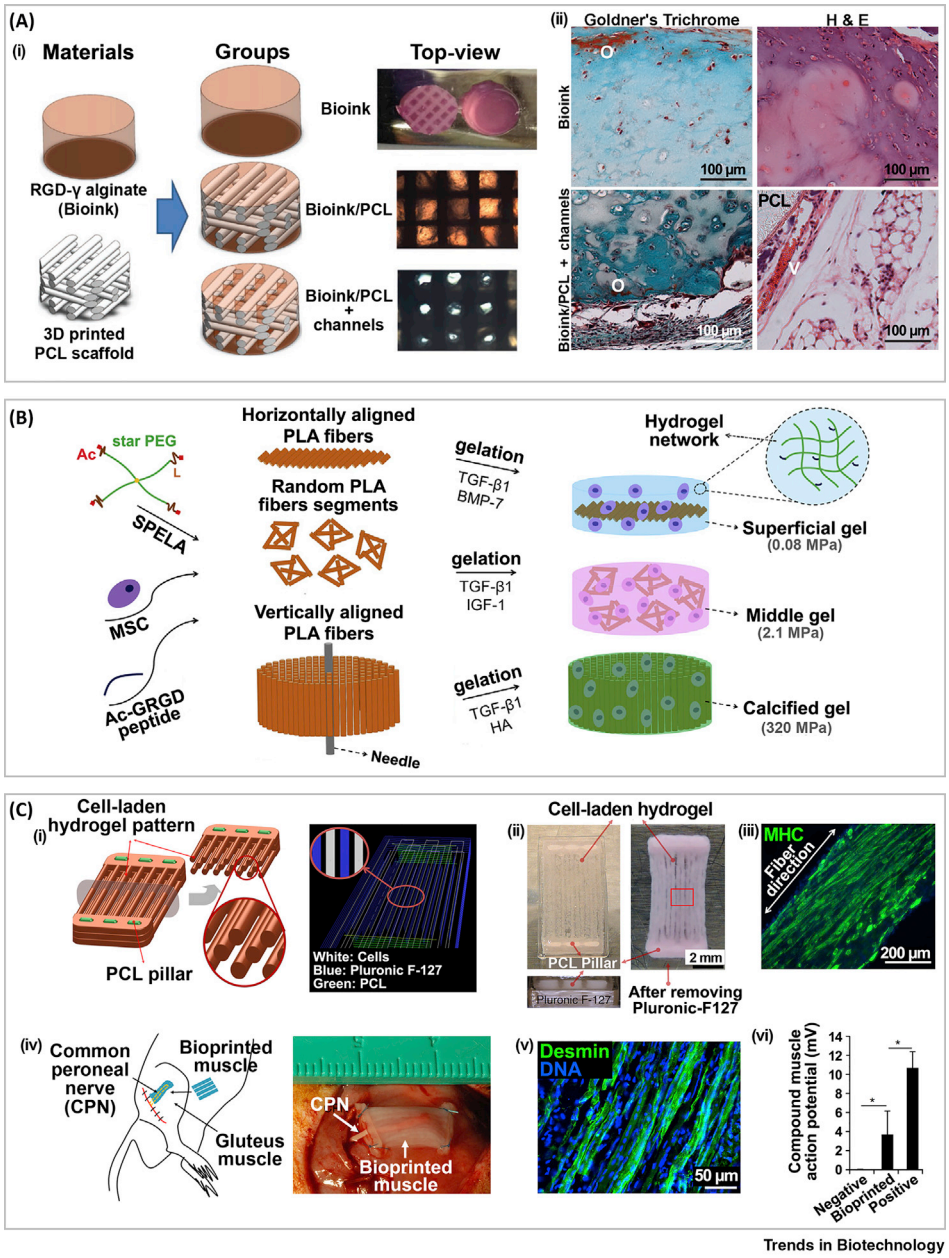


Figure 2. Hybrid Structures in (A) Bone, (B) Cartilage, and (C) Skeletal Muscle Tissue Engineering.

(A) (i) Cell-carrying hydrogels reinforced with poly(ε-caprolactone) (PCL) scaffolds; (ii) histological sections 12 weeks postimplantation. (B) Schematic representation of hydrogels reinforced with electrospun nanofibers to mimic the superficial, middle, and calcified zones of articular cartilage. The fibers at different orientations, the inclusion of hydroxyapatite nanoparticles for the calcified region, and the culture conditions influenced the activity of embedded mesenchymal stem/stromal cells. (C) (i) Designed fiber bundle structure for muscle organization. PCL pillars were used to maintain the structure and to induce the compaction phenomenon for cell alignment; (ii) 3D patterning outcome of designed muscle organization before (left) and after (right) removing the sacrificial material; (iii) immunofluorescent stain (7-day) for myosin heavy chain (MHC) of the bioprinted muscle organization indicated that myoblasts aligned along the longitudinal direction of the fibers; (iv) schematic diagram and subcutaneous implantation of the bioprinted muscle construct with the dissected common peroneal nerve inserted into it; (v) harvested implants showed the presence of organized muscle fibers within

(Figure legend continued at the bottom of the next page.)

and calcified zones, the physical cues had a dominating effect on the differentiation of MSCs, specifically the zone-specific matrix modulus.

Skeletal and Cardiac Muscle

The highly organized skeletal muscle tissue is composed of myofibers, nerves, vascular networks, and extracellular connective tissue, and has intrinsic capacity to regenerate [31]. Myoblasts (differentiated satellite cells) fuse together to form elongated and multinucleated structures called myotubes. These mature to form myofibers, the basic structural unit of skeletal muscle, which are supported by connective tissue and create parallel aligned bundles [32].

Hybrid 3D structures can improve the development of skeletal muscle tissue by properly replicating its architecture and imparting electrical conductivity, which has been described as fundamental for proper muscle tissue development [33]. Examples of hybrid constructs used for this purpose include incorporation of nanoparticles within hydrogel for the fabrication of mechanically robust and electrically conductive nanocomposites [34] and/or the incorporation of aligned fibers within hydrogels to provide internal patterning and guidance for muscle cells [15], emulating its native architecture. Ahadian and coworkers [34] studied the effect of incorporating **carbon nanotubes (CNTs)** with different spatial configurations within gelatin-based hydrogels on the myogenic activity of skeletal muscle cells. The hydrogel with aligned CNTs presented anisotropic electrical conductivity when compared with pristine hydrogels or hydrogels containing randomly distributed CNTs. Cells cultured on aligned CNTs hydrogels yielded a higher number of functional myofibers than cells cultured on hydrogels with randomly distributed CNTs and horizontally aligned CNTs. Additionally, the myogenic activity was more pronounced after applying electrical stimulation along the direction of aligned CNTs, due to anisotropic conductivity of the hybrid construct with vertically aligned CNT.

Using a different strategy, Kang and coworkers [15] (Figure 2C) explored the mimicry of skeletal muscle tissue architecture via the biofabrication of hydrogel filaments containing C2C12 cells together with sacrificial filaments and PCL pillar structures. The sacrificial filaments served as support for the hydrogel during printing, while the PCL pillars further aided the stabilization of 3D bioprinted muscle-mimetic organization. More specifically, they induced compaction of patterns of cell-laden hydrogel towards cell alignment in a longitudinal direction of the bioprinted constructs, which was not observed in 3D hybrid systems without PCL support. Muscle constructs retrieved from subcutaneous implantation in nude mice presented well-organized muscle fiber structures, nerve (neurofilament) contacts, vascularization, and action potential (a trigger event that leads to contraction).

Although cardiac and skeletal muscle tissues share some functional and anatomic features, they respond quite differently to injury [35,36]. Despite its relevance as a vital organ, the regenerative capacity of the heart in adult organisms is very limited. Normal cardiac wound-healing produces collagen-rich scars that eventually undergo maturation in a (currently considered) irreversible process and this nonfunctional scar tissue hampers proper myocardium regeneration [35,36]. One of the primary goals in cardiac TERM is the recovery of the heart beating/pumping function. This is quite challenging as myocardium is a complex tissue with specialized vascular structure and function, specific electrical conduction, high metabolic demand, great compliance, and particular ability to rapidly adapt to external demands [35,36].

the implanted construct; and (vi) the functional assessment showed that they responded to electrical stimulation to an extent consistent with immature/developing muscle after 4 weeks of implantation. (A) (i,ii) Reproduced/adapted from [16], with permission; (B) reproduced/adapted from [28], with permission; (C) (i–vi) reproduced/adapted from [15], with permission. Abbreviations: Ac, Acrylate group; BMP-7, bone morphogenetic protein 7; HA, hydroxyapatite, IGF, insulin growth factor; L, lactide segments; O, osteoid; PCL, poly(ε-caprolactone); PEG, poly(ethylene glycol); PLA, polylactide; Ac-GRGD, acrylamide-terminated glycine-arginine-glycine-aspartic acid; SPELA, star acrylate-terminated lactide-chain-extended polyethylene glycol macromer; TGF-β1, transforming growth factor β1; V, vessel formation.

The combination of structures to impart different properties to 3D TE constructs has been assessed in a few studies to better fulfill some of the cardiac tissue engineering demands [17,37]. For example, hydrogels were reinforced with particles that enhanced their electrical performance and mechanical strength. The hybrid systems promoted improved rhythmic contraction by rat neonatal cardiomyocytes. In a more complex system, Wu and colleagues [38] (Figure 3A) successfully mimicked cardiac anisotropy by using aligned conductive nanofiber yarn networks of PCL/CNTs/silk fibroin embedded within a gelatin-based hydrogel. By using these hybrid structures, cardiomyocyte orientation and maturation could be controlled. More interestingly, an endothelialized myocardium could be obtained by coculturing cardiomyocytes and endothelial cells (ECs) within the 3D hybrid structure.

Nerve

Throughout the years, research in neural tissue engineering has been mainly directed towards the fabrication of nerve-graft replacements for treatment of peripheral nerve injuries. The regenerative ability of the peripheral nervous system is higher than that of the central nervous system (CNS) [39], mainly due to differences in the response to injury of the corresponding neuroglial cells [39].

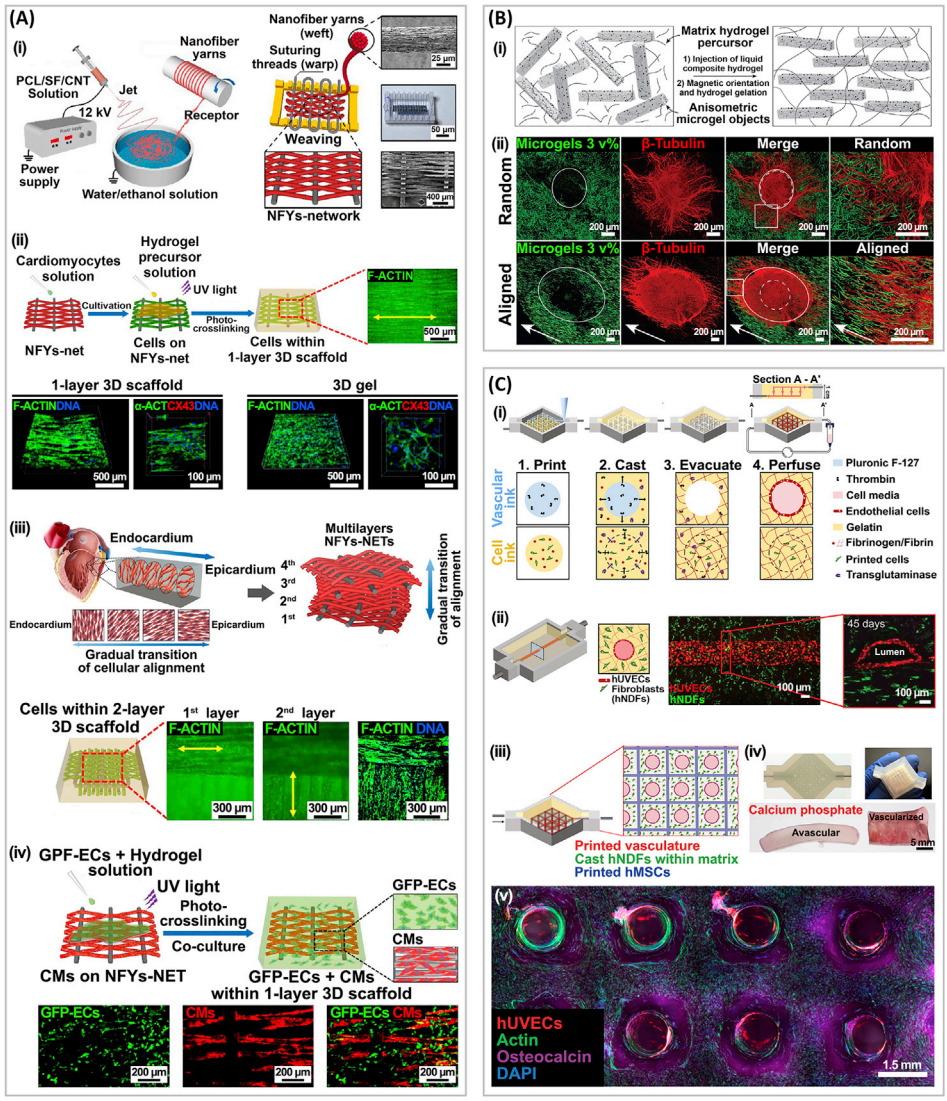
The functionality of peripheral nerves is compromised when these are injured by transections or crushes. In some cases, the proximal and distal portions of the nerve maintain continuity, facilitating the regrowth of transected nerves. However, others produce extensive gaps that are difficult to bridge [40]. The main goal of nerve TERM strategies is to provide electrically permissive and mechanically stable microenvironments, with defined architecture, capable of bridging regenerating axons [40].

Hybrid 3D structures designed for nerve regeneration share similar features to those developed for muscle TERM. CNTs have been used to impart electroactive properties to collagen-based [41,42] or poly(2-hydroxyethyl methacrylate) [43] hydrogels, which influence the cellular activity. Koppes and colleagues [42] incorporated single-walled CNTs within collagen type I hydrogels. Neurite outgrowth from embedded dorsal root ganglia was promoted by either electrical stimulation or inclusion of the CNTs, but the combination of both stimuli significantly increased the neurite outgrowth when compared with each cue alone.

Incorporation of spatial guidance by means of internal topography tailoring has also proven to be beneficial for improved outcomes in nerve TERM. This can be achieved via spatial arrangement of particles [44] and rods [45], or by incorporating aligned nanofibers within the hydrogel [46]. Rose and coworkers [45] (Figure 3B) explored the use of injectable hybrid hydrogel incorporating rod-shaped, magnetoceptive, soft microgels (prepared by mold-based soft-lithography) within an even softer hydrogel. Microgels were aligned applying a magnetic field and the liquid surrounding hydrogel precursor solution was crosslinked to fix the microgel orientation. Dorsal root ganglia were positioned within hydrogels containing random or magnetically aligned microgels and β-tubulin staining revealed neurite outgrowth parallel to aligned microgels. The use of a different type of reinforcing structure, such as PCL aligned nanofibers, has also been assessed [46]. These were incorporated within hyaluronic acid hydrogels and significantly increased both the number of oriented neurites and the distance at which they could extend.

Vascularization

One of the main challenges with the clinical application of TE constructs is their functional integration in the host tissue, which is highly dependent on adequate and timely vascularization. Natural vascular networks form mainly via two mechanisms: **vasculogenesis** and **angiogenesis** [47–49]. Upon implantation, vascularization of TE constructs can take days or weeks, and those with anatomically relevant size typically hinder the diffusion of oxygen and nutrients. Consequently, if not rapidly vascularized, the inner parts of the TE constructs may undergo necrosis, ultimately resulting in implant failure [47,48]. Therefore, it is highly important to decrease the time that a TE construct takes to be vascularized upon implantation through proper scaffold design [47,48]. This should ensure that all cells are provided with nutrients and oxygen, meaning that cells should be within a distance of 100–200 μm from a vessel, generally regarded as the diffusion limit within a tissue [47,48]. Different



Trends in Biotechnology

Figure 3. Hybrid Structures in (A) Cardiac and (B) Nerve Tissue Engineering and for (C) (Pre)vascularization of Tissue Engineered Constructs.

(A) (i) Schematic illustrations of the nanofiber yarns fabrication. (ii) Cardiomyocyte (CM) seeding/culture and corresponding fluorescent images. Confocal images showed CM alignment and elongation within a one-layer 3D scaffold and random morphology within hydrogel. (iii) Fabrication of nonaligned hybrid scaffolds mimicking the myocardium tissue anisotropy. (iv) Schematics showing the coculture procedure of CMs seeded on the yarns network layer and endothelial cells (ECs) encapsulated within hydrogel. (B) (i) Microgels (rod-shaped, magnetoceptive) are aligned and their orientation fixed *in situ* by applying a magnetic field and crosslinking the softer hydrogel precursor solution. (ii) β-Tubulin staining revealed neurite outgrowth parallel to the aligned microgels in contrast with randomly distributed microgels. (C) (i) Schematic illustrations of the fabrication process. (ii) A single EC-lined vascular channel supporting a fibroblast cell-laden matrix and housed within a 3D perfusion chip and (iii) the geometry of the multichanneled printed heterogeneous tissue. (iv) Photographs of a printed tissue construct and cross-sections of avascular/vascularized tissue after 30 days of osteoinductive media perfusion (alizarin red stain). (v) Confocal microscopy image through a cross-section of vascularized tissue construct after 30 days of active perfusion and *in situ* differentiation. (A) (i–iv) Reproduced/adapted from [38], with permission; (B) (i,ii) reproduced/adapted from [45], with permission; (C) (i–v) reproduced/adapted from [54], with permission. Abbreviations: CNT, Carbon nanotube; GFP, green fluorescent protein; hMSCs, human mesenchymal stem cells; hNDF, human neonatal dermal fibroblasts; hUVEC, umbilical vein endothelial cells; NFY, nanofiber yarns; PCL, poly(ε-caprolactone); SF, silk fibroin.

strategies have been used to promote prevascularization of TE constructs. Most exploit the intrinsic blood vessel-forming ability of ECs and often combine them with mural cells to improve the stabilization and maturation of prevascular structures [50,51]. However, very little is known regarding the impact of structural reinforcement of cell-laden hydrogels on the development of vascular networks. Recently, Li and colleagues [52] reported the development of an injectable hybrid system of hyaluronic acid hydrogel reinforced with PCL electrospun nanofibers, able to generate tubular endothelial structures with lumen and branching. Such structures were not observed within hydrogels alone with similar mechanical properties. The presence of the microfibers was shown to promote improved biological outcomes, by providing spatial guidance to cells towards vasculogenesis within hydrogels.

3D hybrid scaffolds have also been particularly useful to increase the size and complexity of prevascularized TE constructs using spatial patterning. Miller and colleagues [53] printed a rigid template as 3D filament networks of a sacrificial carbohydrate glass. This lattice was further embedded within different cell-carrying hydrogels. The entrapped 3D network was then dissolved in cell culture media, yielding a tissue construct with a vascular architecture matching the original lattice. Human ECs and cells from a mouse embryonic cell line were able to surround the void spaces, endothelializing the channel walls. Using a similar principle, but increasing the complexity, volume, and culture time, a perfusable, endothelialized vascular network within 3D matrices was created by Kolesky's team [54] (Figure 3C). The system supported the culture of human ECs, dermal neonatal fibroblasts, and MSCs, and the differentiation of MSCs towards the osteoblastic lineage could be observed after 30 days of culture.

Concluding Remarks and Future Perspectives

In the development and optimization of 3D structures for TERM, only recently has the focus shifted from biomaterials and their structural features toward biological components/processes. As the dynamics of tissue morphogenesis is better understood, the design of TE scaffolds is becoming more centered on cellular activity. As such, it is currently well recognized that, more than being mere surrogate structures, TE constructs should be designed to orchestrate the spatial/temporal dynamics of tissue formation/regeneration. The way that cells behave when entrapped or recruited towards hydrogel-based 3D matrices provide several clues for the improved design of these artificial microenvironments. Although these systems are evolving, they are still quite simple compared with their *in vivo* counterparts, especially concerning dynamic changes of native tissues. The recognized limitations of hydrogels have propelled the development of hybrid systems. The incorporation of different structures, like porous scaffolds, fibers, CNTs, or/and magnetic particles, within hydrogels impart features that they cannot provide alone. Interestingly, as some studies indicate [28,52], the reinforcement of hydrogels using biophysical factors may even replace/surpass the effects of biochemical cues, which, for some applications, would be highly beneficial in order to lower or even eliminate the amount/type of transient biochemical cues required.

While the therapeutic use of hybrid systems faces regulatory approval stages like other biomaterials-based TERM strategies, the fact that most studies with such systems have been essentially using components made of already approved materials (e.g., PCL, hydroxyapatite, alginate, hyaluronic acid) may help accelerate the process. Furthermore, they present some potential advantages, such as longer shelf-life and lower production costs (compared with biochemical factors). Although cost may be difficult to predict, biophysical factors are expected to be simpler and less expensive than biological agents, especially at industrial-scale production levels.

The development of hybrid 3D systems faces the same hurdles of other TE structures (see Outstanding Questions). They should ideally provide adequate (bio)physical-chemical cues, in a spatiotemporal manner, to foster new tissue formation and proper maturation, towards the design of clinically relevant structures. The success of these structures is strongly linked to the hydrogel/reinforcing structure and the cell/hybrid system interactions. However, there is no preferred or universal type of reinforcement. Each tissue has its own specificities, which may eventually be more closely recapitulated by combining different properties/types of reinforcements into a single construct. This can also improve the mimicry

of the structural internal organization and stratification of native tissues and, depending on the tissue, facilitate the successful incorporation of more than one cell type.

Most of the studies conducted so far (Table 1) focused on musculoskeletal and electroactive tissues such as bone, cartilage, muscle, and nerve. Hybrid systems that mimic load-bearing tissues are the most studied, probably because the increase of overall stiffness of a construct by simple incorporation of microfibers or particles is a straightforward and relatively easy property to achieve. However, the parallel evolution of processing techniques and fundamental biological understanding of regenerative processes will certainly increase the diversity of target tissues. In fact, the rapid evolution and progress in processing technologies such as biofabrication will certainly help to develop more complex and highly organized structures, such as skin-, gut-, kidney-, or lung-like tissues, with specific internal architectures and clinically relevant sizes. Among these, skin, kidney, or lung tissues are examples of tissues that have not yet been targeted with hydrogel-based hybrid systems but arise as strong candidates. However, the concomitant increased knowledge on tissue morphogenesis, and the design of 4D strategies that consider the spatial/temporal dynamics of such processes, will certainly contribute to the design of more efficient and clinically relevant hybrid systems.

Outstanding Questions

Will hybrid systems advance the TERM field by more adequately mimicking complex tissue-specific features at multiple scales?

Will it be technically challenging to evaluate the effects of such complex structures and their biochemical/biophysical properties on cell response dynamics, at different levels?

Will potentially complex hybrid systems be easy to scale-up and to translate into the clinics and will they keep their biofunctionality for the required timeframe?

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