

Hierarchical Design of Tissue-Mimetic Fibrillar Hydrogel Scaffolds

Alberto Pardo, Manuel Gomez-Florit, Matthew D. Davidson, Meftune Özgen Öztürk-Öncel, Rui M. A. Domingues,* Jason A. Burdick,* and Manuela E. Gomes*

Most tissues of the human body present hierarchical fibrillar extracellular matrices (ECMs) that have a strong influence over their physicochemical properties and biological behavior. Of great interest is the introduction of this fibrillar structure to hydrogels, particularly due to the water-rich composition, cytocompatibility, and tunable properties of this class of biomaterials. Here, the main bottom-up fabrication strategies for the design and production of hierarchical biomimetic fibrillar hydrogels and their most representative applications in the fields of tissue engineering and regenerative medicine are reviewed. For example, the controlled assembly/arrangement of peptides, polymeric micelles, cellulose nanoparticles (NPs), and magnetically responsive nanostructures, among others, into fibrillar hydrogels is discussed, as well as their potential use as fibrillar-like hydrogels (e.g., those from cellulose NPs) with key biofunctionalities such as electrical conductivity or remote stimulation. Finally, the major remaining barriers to the clinical translation of fibrillar hydrogels and potential future directions of research in this field are discussed.

(GAGs), proteoglycans) that constitute the intercellular space. Besides providing structural support for cells to form tissues, the ECM also supplies them with biochemical (soluble macromolecules) and mechanical cues that direct cell growth and phenotype.^[1] Moreover, cells contribute to constant ECM maintenance and remodeling.^[2]

The main building blocks of native ECMs are collagen, elastin, and fibronectin, which have nano-to-microscale fibrillar structures.^[3] These ECM molecules (nanoscale) are hierarchically assembled into fibrils (nano-to-microscale), fibers (microscale), and fiber bundles (micro-to-macroscale), to form complex 3D fibrillar networks (Figure 1a).^[4] The structure and biochemical makeup of these networks strongly influence ECM physical properties, such as their stiffness, plastic

deformation,^[5] and/or strengthening,^[6] to provide each tissue with a unique nonlinear (viscoelastic) mechanical behavior.^[7,8]

Due to their markedly different mechanical properties, the relative amounts of individual ECM components and their density,

1. Introduction

The extracellular matrix (ECM) is a fibrillar network of insoluble macromolecules (proteins, glycoproteins, glycosaminoglycans

A. Pardo, M. Ö. Öztürk-Öncel, R. M. A. Domingues, M. E. Gomes
3B's Research Group I3Bs – Research Institute on Biomaterials
Biodegradables and Biomimetics University of Minho Headquarters of
the European Institute of Excellence on Tissue Engineering and
Regenerative Medicine AvePark – Parque de Ciência e Tecnologia Zona
Industrial da Gandra Barco
Guimarães 4805-017, Portugal
E-mail: rui.domingues@i3bs.uminho.pt; megomes@i3bs.uminho.pt

A. Pardo, M. Ö. Öztürk-Öncel, R. M. A. Domingues, M. E. Gomes
ICVS/3B's - PT Government Associate Laboratory
Braga/Guimarães 4710-057, Portugal

A. Pardo
Colloids and Polymers Physics Group
Particle Physics Department
Materials Institute (iMATUS)
and Health Research Institute (IDIS)
University of Santiago de Compostela
Santiago de Compostela 15782, Spain

M. Gomez-Florit
Health Research Institute of the Balearic Islands (IdISBa)
Palma 07010, Spain

M. Gomez-Florit
Research Unit, Son Espases University Hospital (HUSE)
Palma 07010, Spain

M. Gomez-Florit
Group of Cell Therapy and Tissue Engineering (TERCIT)
Research Institute on Health Sciences (IUNICS)
University of the Balearic Islands (UIB)
Ctra. Valldemossa km 7.5, Palma 07122, Spain

The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/adhm.202303167>

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alignment, and thickness, among other factors, determine the properties and function of each tissue (Figure 1b).^[9] Collagen fibers impart stiffness and strength to tissues, elastin forms extensible fibers that undergo high levels of deformation and elastically recoil the tissue back to an original shape,^[10] and fibronectin is arranged into stretchable fibers that facilitate cell adhesion and migration through interactions with integrins.^[11] For example, the highly aligned collagen-rich architecture of tendons is responsible for the stretched morphology of tenocytes, the non-linear elasticity of the tissue, and the efficient energy transfer between muscle and bone during locomotion.^[12,13] In contrast, the ECM of bone is highly mineralized, introducing a higher stiffness to the tissue for load-bearing processes.^[14]

Considering the fundamental role of ECM composition and architecture for tissue integrity and functionality, its disruption under pathological circumstances alters tissue function (Figure 1c).^[15] For example, during tendon fibrosis and/or scarring, fiber thickness and density are increased,^[16] which leads to pathological tissue properties.^[17,18] A greater stiffness of the ECM has also been observed during aging and is attributed to increased crosslinking of collagen fibers, which compromises tissue function.^[19] Increased fiber diameter, ECM alignment, and stiffening are also evident in tumor stromal tissue and are believed to facilitate metastasis.^[20,21]

Beyond altering the macroscopic tissue properties, the fibrillar nature of ECMs also modulates biological mechanotransduction, the set of processes by which physical forces are sensed and converted into biochemical and electrical signals that impact cellular behavior.

Motivated by the importance of ECM structure and implications in tissue properties and cellular behaviors, this review describes common design strategies to fabricate biomimetic hydrogel-based systems that replicate the complex hierarchical fibrillar architecture of tissues. Further, the review ends with a discussion of the limitations of current systems and provides our perspectives on future developments in the field.

2. Mechanotransduction Pathways: Biophysical Cues over Cells Interacting with Biomimetic Fibrillar Hydrogels

The interaction of resident cells with native ECMs takes place through specific surface receptors and induces numerous intracellular signaling pathways that regulate gene expression and ultimately dictate cell behavior. ECM fibers bind and retain growth factors, a mechanism that is often critical for their effective presentation to cell surface receptors and downstream biological signaling.^[22]

Cells can detect changes in the mechanical properties of the surrounding ECM by applying traction forces on single fibers,

thus triggering the mechanotransduction signaling mechanisms that change cell phenotype.^[23] In addition, cell-ECM mechanical linking through fibrillar networks allows them to rapidly transmit forces over long distances, thus activating signaling pathways and providing positional information about other cells within the ECM.^[24] The major mechanosensing pathways to detect and respond to biomechanical stimuli from fibrillar ECM are transmembrane receptor molecules (e.g., integrins), ion channels (being particularly relevant PIEZO channels, that convert mechanical stimuli into electrochemical signals), and cytoskeletal components such as actin filaments that transduce external cues directly to the nucleus via integrins.^[25] When triggered, these mechanosensors prompt responses that govern cell fate, such as cytoskeleton reorganization, ion fluxes, the activation of the Rho family of GTPases (RHO) and mitogen-activated protein kinases (MAPK), and the release of extracellular adenosine triphosphates (ATP) that induce inward currents and the depolarization of the cells (Figure 2a).^[26]

Besides their general fibrillar structure, other biophysical properties of engineered fibrillar-hydrogels strongly dictate the fate of encapsulated/seeded cells. For instance, the stiffness of cell-laden fibrillar scaffolds has an important influence over the growth, proliferation, and migration of cells,^[27] while their (anisotropic) topography and/or porosity also impact the morphology and migration of cells.^[28] Other important characteristics of native ECMs, such as their complex viscoelastic stress-relaxation behavior, also have an essential role in controlling cell behavior in artificial scaffolds.^[29]

Moreover, native resident cells also experience exogenous forces (e.g., shear stress and interstitial flow-derived forces caused by blood flow and draining lymphatics) that may be replicated in engineered constructs.^[30] External forces such as these may also change fibrillar ECM properties to stiffen them, which can alter cell morphology and reorganize their cytoskeleton (Figure 2b).^[31] All of these biophysical cues must be carefully considered to design hydrogels that provide cells with signals related to mechanotransduction.

Providing hydrogels with biomimetic fibrillar structures is essential to replicate these phenomena in biomaterials either designed for tissue engineering (TE) purposes or to model features of disease. After an injury or a disease, this intricate ECM organization is disrupted, leading to a natural healing response of the body that consists of the formation of new scar tissue that exhibits a disorganized structure and different functionalities from the original native tissue.^[32] Thus, engineered constructs should replicate the architecture of their native counterparts in both health and disease, generating biological microenvironments that provide cells with adequate biophysical and biochemical cues during in vitro culture and in vivo after implantation.^[33] Fortunately, the generation of fibrillar structures within hydrogels results in highly diverse materials due to widespread chemistries and fabrication tools available, to impart signals to cells that are readily encapsulated within or seeded atop the materials.^[34,35]

3. Assembly Approaches to Fabricate Fibrillar Hydrogels

A wide range of strategies have been proposed for the fabrication of hydrogels with fibrillar-like architectures. Of particular

M. D. Davidson, J. A. Burdick
BioFrontiers Institute and Department of Chemical and Biological Engineering
University of Colorado Boulder
Boulder, CO 80303, USA
E-mail: jason.burdick@colorado.edu

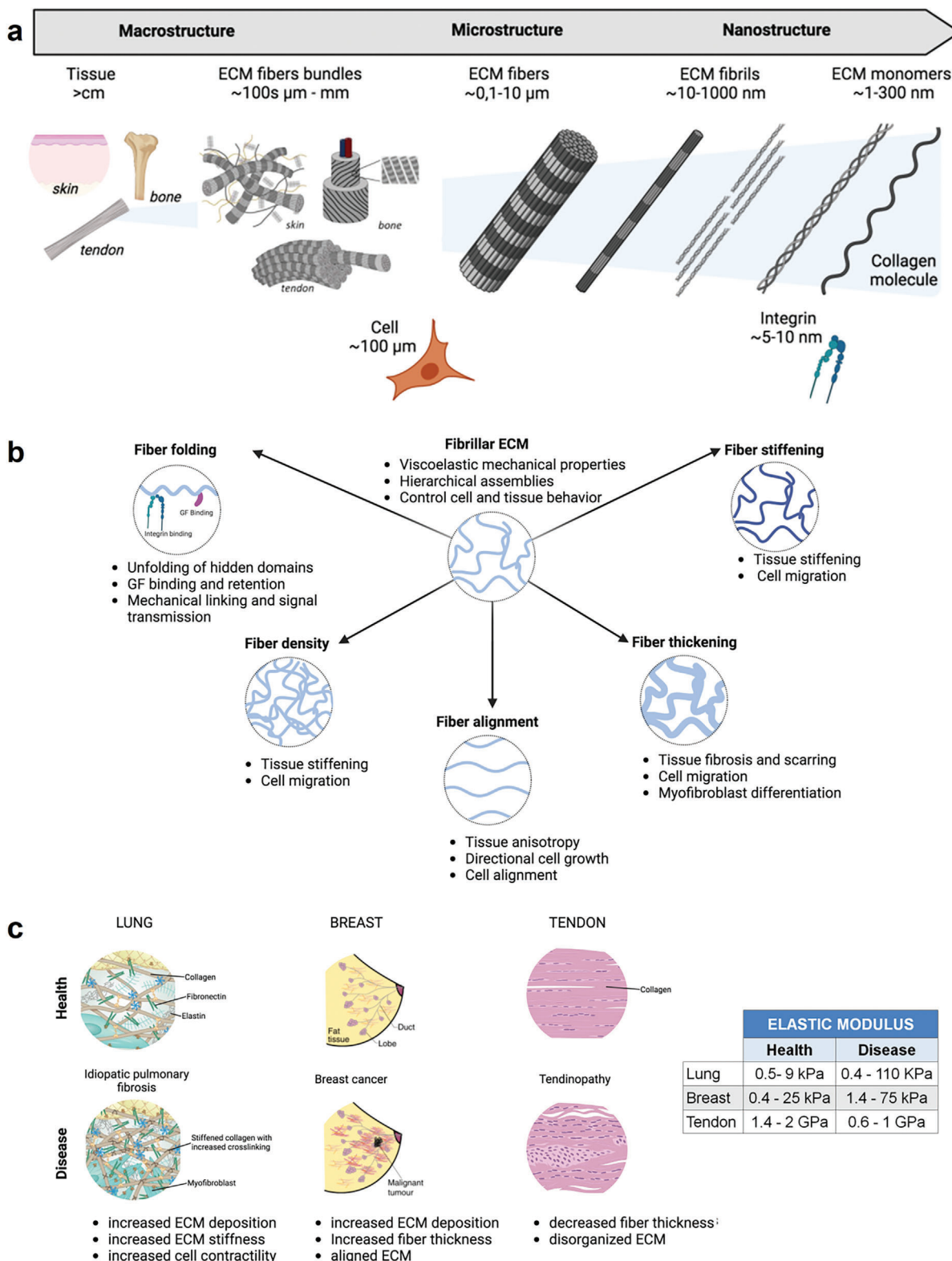


Figure 1. Role of fibrillar structures in extracellular matrices. a) Individual ECM molecules assemble into hierarchical and fibrillar macromolecules that provide each tissue with specific mechanical properties at various length scales. To exemplify this, the self-assembly of collagen is represented. From the molecular level, three collagen polypeptide chains form a triple helix (tropocollagen). Multiple tropocollagens bundle together into protofibrils, alternating gap and overlapping regions, which are visible in transmission electron microscopy images as repeating light (gap region) and dark (overlap region) striations with a spatial period of ≈ 67 nm. The protofibrils further bundle together into collagen fibrils, which form collagen fibers and fiber

interest are the bottom-up approaches, which allow the manufacturing of fibrillar hydrogels with highly controlled structures through the assembly of different typologies of nano- and macro-building blocks. Moreover, some of these building blocks can also be used as nano- or micro-fillers to generate a desired fibrillar feature within hydrogels.

The characteristics of the building blocks/fillers used to fabricate fibrillar hydrogels strongly dictate the dimensions and other physicochemical properties of the constituent fibers, which allows the engineering of a wide range of native ECMs. For example, hydrogels based on the assembly of nano-building blocks such as peptides or block copolymer micelles form fibrils with diameters in the range of a few tens of nm, approaching the dimensions of reticular collagen fibers found in the fibrous reticulum of lymphoid tissues.^[36] Thus, these systems have found applica-

tion in the engineering of tissues such as bone marrow, spleen, or liver.^[37,38]

The use of micro-building blocks, such as electrospun microfibers or polymeric microgels, allows the design of hydrogels with fibrillar diameters in the micrometer range. These hydrogels have been evaluated for the engineering of tendons, ligaments, or skeletal muscles since the micrometric fibrillar structures closely match the dimensions of collagen fibers present in native tissues.^[39] Although the dimensions of the generated fibers are an important parameter, the choice of building block also affects the biomechanical properties of the final constructs. For this reason, the selected fabrication method must consider the stiffness, viscoelasticity, permeability, and/or swelling degree of the intended tissue, among many other properties. For example, fibrillar hydrogels based on the controlled self-assembly of

bundles that constitute tissues. Adapted with permission.^[210] Copyright 2021, Frontiers, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>). b) Changes in fiber stiffness, thickness, alignment, density, and folding impact the physical properties of the ECM and modulate biological signaling. c) Variations in ECM fibrillar structure and the elastic moduli of the lung,^[211] breast fat tissue,^[212] and tendon^[17,18] between healthy and diseased states. Schematic of lung adapted with permission.^[213] Copyright 2017, European Respiratory Society; schematic of breast tissue adapted with permission.^[214] Copyright 2020, Springer Nature, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

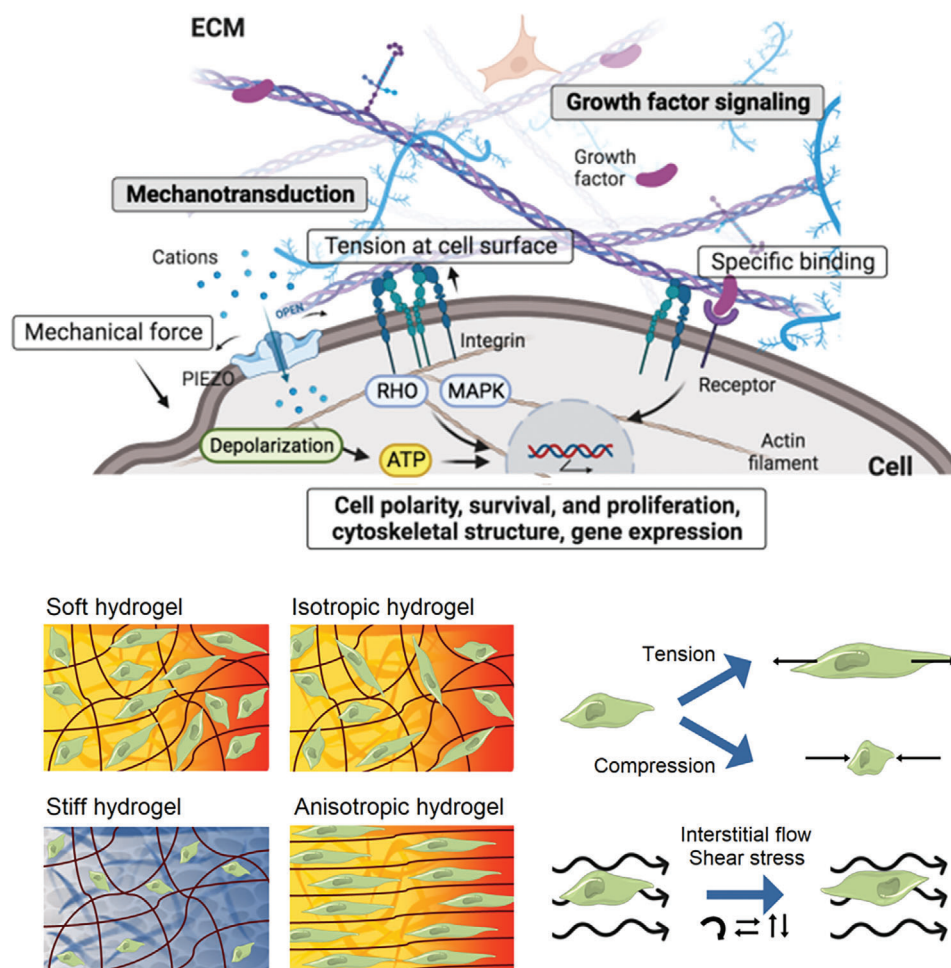


Figure 2. a) Representation of the most relevant mechanosignaling mechanisms involved in the interaction between fibrillar ECMs and resident cells. b) Schematic representation of the effect of stiffness and structural anisotropy of biomimetic fibrillar hydrogels and external forces over cell behavior.

NANOBUILDING BLOCKS AND NANOFILLERS

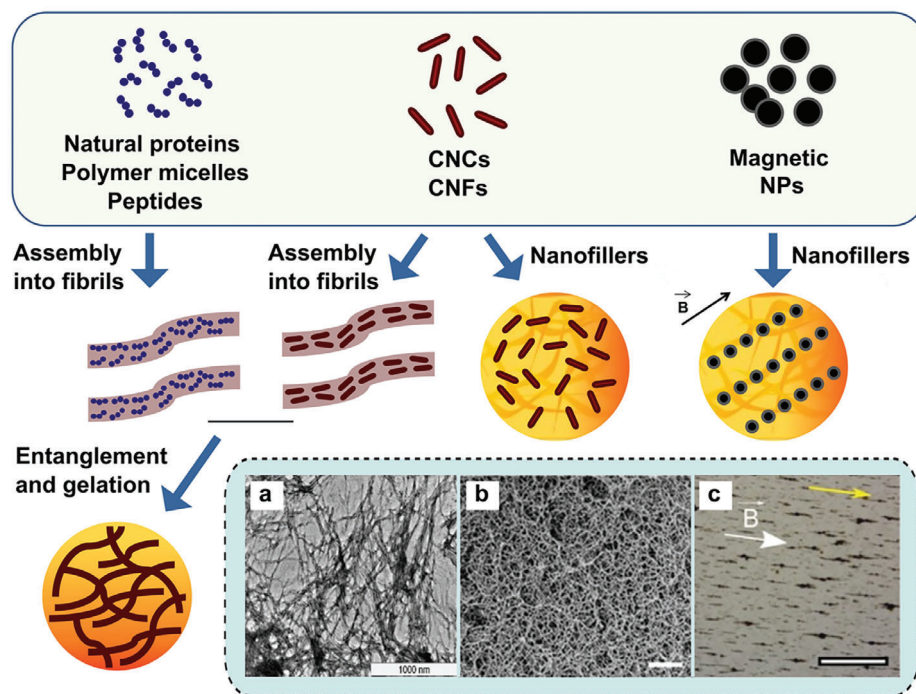


Figure 3. Bottom-up fabrication strategies of fibrillar-like hydrogels based on nano building blocks/nanofillers. Natural proteins, peptides, block copolymer micelles, and cellulose nanocrystals/nanofibers can self-assemble into fibrils and then entangle to form fibrillar hydrogels. On the other hand, cellulose nanostructures and magnetic nanoparticles can be used as nanofillers to provide hydrogels with fibrillar architectures. In the bottom are shown representative microscopy images of fibrillar-like hydrogels consisting of a) self-assembled tetrapeptide Asp-Leu-Ile-Ile. Reproduced with permission.^[83] Copyright 2022, American Chemical Society; b) self-assembled rod-shaped cellulose nanocrystals. Reproduced with permission.^[102] Copyright 2021 Wiley-VCH; c) MNPs forming chains within GelMA hydrogels under applied external magnetic field. Reproduced with permission.^[110] Copyright 2019, Wiley-VCH.

short peptides display low storage moduli and may be important for the engineering of soft tissues, whereas fibrillar hydrogels based on cellulose nanocrystals typically result in stiffer constructs and may be more appropriate for stiffer tissues.

Despite the wide range of fibrillar hydrogel properties that can be controlled by varying the building blocks, hydrogels often are limited in their potential to replicate the complexity of native tissues, which can be mitigated by the additional incorporation of nanostructures into their design. For example, the addition of ceramic nanoparticles has been widely explored to enhance the mineralization degree of fibrillar hydrogels for bone TE purposes. Similarly, magnetic and conductive nanoparticles have been used to provide the scaffolds with remote magnetic responsivity and electrical conductivity, respectively.^[40]

In this section, we discuss the main bottom-up fabrication strategies based on nano- (Figure 3) and micro-building blocks (Figure 4) used in the design of biomimetic hydrogels with fibrillar-like hierarchical structures. These strategies are described in the context of specific applications.

3.1. Nano-Building Blocks and Nanofillers

3.1.1. Natural Fibrillar Proteins

The production of hydrogels exclusively composed of natural fibrillar proteins has been widely reported in the biomedical

field.^[41] These biomaterials are readily degradable and maintain part of the biochemical complexity of native tissues, displaying an inherent bioactivity that enables cell adhesion, proliferation, and remodeling.^[42] However, limitations such as their low structural stability and the difficult modulation of their mechanical properties limit the potential of protein hydrogels. Collagen-based hydrogels can be easily prepared from acidic collagen type I solutions after pH adjustment and thermo-induced gelation at physiological conditions. Extrusion^[43] or freeze-drying techniques,^[44] among others, have been proposed as methods to develop collagen hydrogels from colloidal solutions. On the other hand, fibrin hydrogels are typically formed through thrombin-mediated enzymatic polymerization of fibrinogen macromolecules, which leads to the fast formation of 3D fibrillar networks.^[45]

These types of constructs have been widely evaluated for 3D cell culture applications. For instance, collagen type I hydrogels have been used as matrices for the 3D culture of fibroblasts^[46] or myoblasts,^[47] constituting a remarkable improvement over traditional 2D cell culture methods and 3D hydrogels that lack nano/microscale fibrillar structures. Interestingly, it was demonstrated that the application of tensile stress to collagen^[48] or fibrin^[49] gels induced the preferential orientation of their fibers to form anisotropic hierarchical hydrogel matrices that replicate the native ECM anisotropy of tendons, ligaments, and menisci. The intrinsic weak diamagnetic response of both collagen type I

MICROBUILDING BLOCKS AND MICROFILLERS

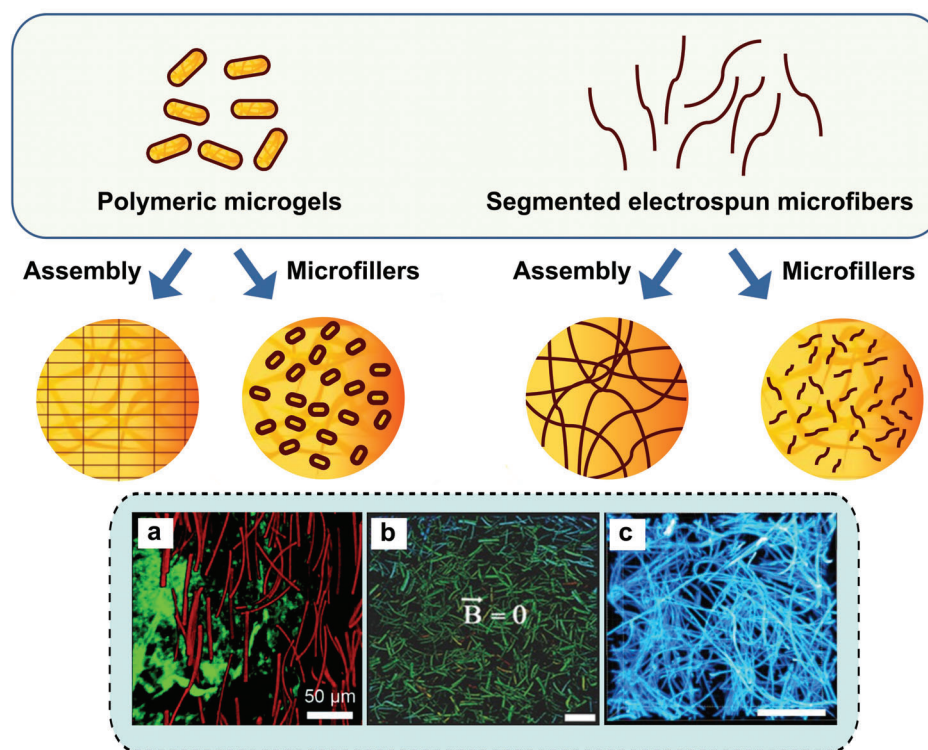


Figure 4. Bottom-up fabrication strategies of fibrillar-like hydrogels based on micro^[223] building blocks/microfillers. Rod-shaped polymeric microgels and fragmented electrospun microfibers can be assembled or used as microfillers to create fibrillar hydrogels. In the bottom are shown representative microscopy images of fibrillar-like hydrogels consisting of a) magnetically-responsive poly(ethylene oxide-propylene oxide) rod-shaped microgels aligned within hydrogels. Reproduced with permission.^[223] Copyright 2020, Royal Society of Chemistry, CC BY-NC 3.0 DEED (<https://creativecommons.org/licenses/by-nc/3.0/>); b) electrospun PLGA microfibers fragmented before their incorporation as microfillers within fibrin hydrogels. Reproduced with permission.^[128] Copyright 2017, Wiley-VCH; c) hyaluronic-acid-based fragmented microfibers assembled and photocrosslinked into highly-porous fibrillar networks. Reproduced with permission.^[132] Copyright 2021, AAAS, CC BY-NC (<https://creativecommons.org/licenses/by-nc/4.0/>).

and fibrin has also been exploited to fabricate anisotropic fibrillar hydrogels, allowing fiber alignment through the application of high-intensity magnetic fields.^[50,51]

Although pure fibrin- and collagen-based hydrogels have been reported for TE applications, the blending of both proteins has also been proposed as an alternative to obtaining constructs with enhanced biomimicry of native tissues.^[52] Other natural proteins derived from purified ECMs or recombinant sources, including elastin, can either be used to directly produce fibrillar hydrogels or mixed with other polymer matrices such as gelatin or silk as reinforcement agents.^[53]

Tissue-specific ECMs secreted by resident cells are also attractive as hydrogel biomaterial sources, allowing close replication of native tissues into bioengineered systems. The most representative example of this strategy is Matrigel, a complex commercially available laminin-dominated protein mixture extracted from Engelbreth–Holm–Swarm mice sarcoma.^[42,54] Matrigel is typically used as a basement membrane matrix for stem cells since it can maintain them in undifferentiated states for long culture times.^[54] However, its tumor-related and variable batch-to-batch composition, together with weak mechanical properties (elastic modulus below 500 Pa), limits its applicability beyond in vitro research studies.

A popular alternative to prepare biomimetic hydrogels based on fibrillar proteins is the decellularization of tissues to obtain decellularized ECM (dECM). In contrast to those based on individual natural ECM proteins, dECM-based hydrogels can retain most of the biochemical and some of the biophysical complexity of native tissues. Moreover, they are typically obtained from healthy tissues, thus overcoming the limitations/concerns of Matrigel due to its tumor origin.^[55] Interestingly, it has been demonstrated that dECM-based hydrogels show different fibril morphology and matrix assembly kinetics when compared to pure collagen type I hydrogels.^[56] The formation of dECM-based hydrogels is based on the self-assembly of collagens regulated by the presence of GAGs, proteoglycans, and other ECM proteins^[56] and then decellularization and solubilization methods that determine the amount and properties of the remaining proteins.^[57] The key roles of ECM components such as heparin, decorin, or fibronectin on dECM-based hydrogels fibrillogenesis have also been demonstrated,^[56,58] highlighting the importance of preserving the biochemical signature of native tissue ECM during the decellularization and solubilization steps. In the most typical approach, dECM is enzymatically solubilized into protein monomeric compounds using pepsin.^[59,60] Afterwards, the spontaneous reformation of the intramolecular bonds of

monomeric components into homogeneous hydrogels is induced by controlling temperature, ionic strength, and/or pH. This strategy was applied to produce dECM-based hydrogels from numerous tissues such as bone,^[61] meniscus,^[62] lung,^[63] or cornea,^[64] among others. Although dECM hydrogels are typically obtained from porcine sources, the use of human tissues has also been reported.^[65]

Despite their promise, hydrogels based on individual ECM proteins or dECM may still fail to provide adequate biophysical cues to encapsulated cells for TE purposes. The application of macromolecular crowding (MMC) has been proposed to more closely replicate the complex structure of native tissues, where the extracellular space is tightly packed with various macromolecules. These macromolecules increase the effective concentrations of molecules and the thermodynamic activity of the system, affecting protein folding, molecular interactions, or enzyme kinetics among other phenomena.^[66] MMC has been widely analyzed for the supplementation of cell culture media, but an understanding of how the incorporation of macromolecular crowders in natural proteins-based hydrogels tailors the desired structural and biophysical hydrogels is still limited.^[67] Pioneering work in this field demonstrated that the incorporation of the artificial macromolecular crowder Ficoll 400 during the assembly of collagen type I hydrogels impacts fiber nucleation, fiber growth, and architecture of the designed constructs.^[68,69] This macromolecule has been also incorporated into kidney dECM-based hydrogels, influencing fibrillogenesis kinetics and the final fibrillar structure and mechanical properties of the obtained scaffolds.^[67] Polyvinylpyrrolidone was also used as a crowding agent in 3D bio-printed collagen hydrogels, facilitating their homogeneous and rapid crosslinking and allowing a higher control over the architecture and porosity of the fabricated 3D collagen matrices.^[70] Other macromolecules such as poly(ethylene glycol), polystyrene sulphate, dextran, and bovine serum albumin have also been evaluated as crowding agents with potential application in the design of fibrillar hydrogels based on natural proteins with enhanced characteristics.^[71]

3.1.2. Assembly of Block Copolymer Micelles

The amphiphilic properties of block copolymer molecules can be exploited to drive their controlled assembly into closed micelles in aqueous solutions.^[72] By varying the molecular weight of the polymer, the relative length of the blocks, or the characteristics of the solvents, it is possible to control the morphology and size of the generated micelles.^[73] On the other hand, when the copolymer concentration exceeds a certain value, the micelles start to arrange themselves into a network and the solution undergoes a transition to the gel state. This transition is typically reversible, having a copolymer concentration-dependent critical temperature below which the system returns to a liquid state.^[74]

Micelles with high aspect ratios (rod-shaped or fiber-like micelles) can be assembled to produce hydrogels with fibrillar structures. In general, worm-like micelles derived from the controlled assembly of block copolymers show widths of a few tens of nm and lengths in the range of several hundreds of nm.^[75] Hydrogels derived from highly-concentrated copolymer solutions are formed by multiple contacts between neighboring micelles,

rather than as a result of a physical entanglement between them. The aspect ratio and the flexibility of the micelles have a remarkable impact on the hydrogel's formation mechanism, particularly when long and low-stiffness micelles act as building blocks.^[75]

Different types of anisotropic micelles based on block copolymers have been evaluated as potential building blocks for designing biomimetic fibrillar hydrogels. For example, poly(2-hydroxypropyl monomethacrylate)-*block*-poly(glycerol monomethacrylate) molecules can be assembled into worm-like micelles at room temperature, which then entangles to form temperature-reversible fibrillar hydrogels at high polymer concentrations.^[76] Similarly, poly(ethylene oxide-*block*-butylene oxide) cylindrical micelles form stiff fibrillar-like hydrogels by temperature and polymer concentration-controlled mechanisms.^[77] Other block copolymers such as poly(ethylene glycol-*block*-2-hydroxypropyl methacrylate)^[78] or poly(methacrylic acid-*block*-styrene-*alt*-N-phenylmaleimide)^[75] have also been proposed for the fabrication of fibrillar hydrogels that recapitulate the architecture of native tissues. Overall, these representative examples demonstrate that block copolymer micelle self-assembly is an easy and tunable route to fabricate hydrogels with fibrillar structures. Moreover, the potential to reversibly change their state and structure through the application of external stimuli (typically temperature change) makes them useful to use in the form of injectable hydrogels. However, the main drawback associated with this type of biomaterials is the potential kinetic effects that can be introduced depending on the selected pathway to induce gelation, especially in the case of high molecular weight block copolymers, which can easily form non-ergodic aggregates.^[79] Additionally, the reduced nanometric scale of the generated fibrillar structures may have a low influence on the behavior of rather larger cellular entities, and the preparation of ordered fibrillar-like hydrogels of relevant clinical size through this approach is still challenging.

3.1.3. Assembly of Peptides

Similarly to block copolymers, peptides can also be used as building blocks to fabricate fibrillar hydrogels. Under specific conditions, peptides can self-assemble into nanometric fibrils that then entangle/branch between them to form fibrillar hydrogels. In terms of fabrication, this class of hydrogels can be prepared by physically assembling the peptides through non-covalent interactions,^[80] or by inducing the chemical^[81] or enzymatic^[82] crosslinking of the building peptide units.

The nature and the number of amino acids in the peptide chains strongly dictate the properties of the formed hydrogels. Thus, the assembly of long-chain peptides typically results in hydrogels with higher mechanical strength than those based on short peptides such as dipeptides or oligopeptides (Figure 3a).^[83] Although the use of natural non-modified sequences of amino acids (e.g., NIFYCPIAIM and IFYCPIAIM ovalbumin peptides) to form fibrillar hydrogels has been reported,^[84] in the most recent approaches peptides are modified with synthetic motifs to control their association and hydrogel formation, as well as to provide specific biofunctionalities. These modifications are an essential design factor, especially in the case of short-chain

peptides that, despite their easier and lower cost synthesis, typically require the subsequent incorporation of additional functional groups in their structure to promote their self-assembly and hydrogel formation.^[85] For instance, the conjugation of short peptides with bulky aromatic groups such as pyrenes or carboxybenzyl can be exploited to control their assembly into different ordered structures, including fibrillar hydrogels. However, the toxicity associated with these functional groups limits the potential biological application of resulting hierarchical constructs. Alternatively, the modification of short peptides with fluorenylmethoxycarbonyl (Fmoc) aromatic groups has been demonstrated to be an efficient strategy to induce their self-assembly into constructs which are especially appealing for biomedical applications due to Fmoc anti-inflammatory properties.^[86] This modification strategy was applied for the fabrication of fibrillar hydrogels based on dipeptides formed by different combinations of phenylalanine, alanine, glycine, and leucine amino acids.^[87]

How encapsulated/seeded cells engage and are affected by the small topographical length scales of the fibrillar structures of peptide-based hydrogels is still unclear. Moreover, the controlled self-assembly of short peptides typically results in weak fibrillar hydrogels (storage moduli below 1 kPa) that have been more widely applied to drug delivery^[88] or biosensing^[89] than TE applications. However, the use of long-chain peptides and/or the incorporation of stiff nanofillers are common design strategies that allow the fabrication of biomimetic hydrogels with stronger mechanical properties as required for the engineering of several tissues. For instance, it was demonstrated that Pro-Asp-(Phe-Asp)₅-Pro peptides self-assemble into β -sheets to form fibrillar hydrogels with potential application for bone TE,^[90] while RADA 16-I peptide-based hydrogels promote the regeneration of damaged cortex and the rejuvenation of aged/degenerated Achilles tendon cells.^[91,92] Further, heparin-binding peptides and KLD tripeptide-based hydrogels were successfully used to promote myocardium and cartilage regeneration, respectively.^[93,94] Remarkably, peptide-based hydrogels are a particularly interesting option for incorporating specific biochemical signals into biomaterial formulations. In representative recent work, peptide fibrillar scaffolds bearing two peptide sequences, one that reduces glial scarring and another that promotes blood vessel formation, enhanced the vascular growth and axonal regeneration of motor neurons.^[95]

3.1.4. Assembly of Cellulose Nanocrystals and Nanofibers

The controlled self-assembly of NPs with adequate physicochemical properties in suspension is an alternative approach to fabricating fibrillar hydrogels. Natural origin NPs with elongated morphologies are especially interesting for this design strategy since they can easily assemble into entangled fiber-like structures that result in biocompatible hydrogels. In this concept, cellulose nanofibers (CNFs) and rod-shaped cellulose nanocrystals (CNCs) are among the most widely explored nanoscale building blocks.

CNFs are long, flexible, and rope-like NPs with both crystalline and amorphous regions, typically extracted from wood products or bacterial cell walls. The diameters of CNFs are below 30 nm, displaying aspect ratios larger than 50.^[96] CNFs can be surface functionalized, for instance, with norbornene groups and then crosslinked through UV- or thermally-initiated thiol-ene click re-

actions to create robust fibrillar hydrogels.^[97] The fabrication of fibrillar hydrogels based on the entanglement and bundling of CNFs has been recently reviewed.^[98] However, CNFs have been more widely explored as reinforcement elements in combination with different polymer matrices than as building blocks to form hydrogels by themselves.^[99]

On the other hand, CNCs are nanosized highly crystalline structures that can be easily obtained from different cellulose-rich sources. They display rod-shaped morphologies, with maximum widths of a few tens of nm and lengths typically up to 300 nm. Their low length and relatively high stiffness hamper the entanglement and bundling of CNCs to form hydrogels, making them more valuable as reinforcement nanofillers.^[100] However, the surface modification of CNCs with temperature-responsive polymers has been proposed to control their physical crosslinking and formation into cytocompatible fibrillar hydrogels.^[101] Moreover, the side-by-side assembling of the CNCs in aqueous suspensions can be induced through different mechanisms which can be controlled by changes in temperature, solution pH, and/or ionic strength, leading to the formation of hydrogels with fibrillar structures when the concentration of nanocrystals is above a critical colloidal limit. The surface charge of CNCs can also be altered with various cations to induce their self-assembly and form biomimetic fibrillar hydrogels from CNC colloidal suspensions, even at low particle concentrations (Figure 3b).^[102]

By varying the charge and ionic radius of the added cations, it is possible to control the mesh size and mechanical properties of the obtained fibrillar hydrogels, thus opening their potential use for TE applications. CNC-based hydrogels have been proposed, for example, to develop in vitro models of biological systems that replicate the complexity of tissue physiology and pathophysiology in miniaturized constructs. For example, fibrillar hydrogels based on self-assembled CNCs were used as a support matrix for embedded 3D bioprinting of vascular channels and their subsequent in situ endothelialization to construct dynamic tumor-on-a-chip models.^[102]

3.1.5. Magnetic Nanoparticle Arrangement to Create Fibrillar-Like Structures

The fabrication of fibrillar biomaterials with anisotropic 3D architectures is an essential design parameter in TE applications since most tissues of the human body display highly organized ECM arrangements.^[103] Although previously discussed building blocks such as CNCs and CNFs can be used as nanofillers to design fibrillar-like hydrogels, controlling their distribution to create this type of anisotropic architecture is still challenging. The incorporation of magnetic nanoparticles (MNPs) into the hydrogel systems has emerged as an especially appealing design strategy to overcome this limitation. MNPs can be remotely assembled/arranged within the hydrogels through the application of non-invasive external magnetic fields, rendering ordered fibrillar-like architectures.^[104,105]

MNPs can be mixed with natural fibrillar protein solutions to direct the development of biomimetic fibrillar hydrogels. For instance, the magnetically-induced unidirectional traveling motion of iron oxide MNPs incorporated within collagen-based hydrogels forces the developed collagen fibers to align in this

same nanoparticle order, creating fibrillar scaffolds with uniaxial anisotropy.^[106] The intrinsic properties of MNPs have been exploited to arrange their distribution within other polymeric hydrogels such as gelatin,^[107] poly(vinyl alcohol),^[108] or alginate^[109] to create biomimetic fibrillar-like composites. The control over the characteristics of the designed MNPs and the applied external magnetic fields allows the formation of magnetic aggregates in the form of linear chains (Figure 3d)^[110] or elliptical clusters,^[108] thus replicating the anisotropic structures of different native ECMs after gelation. In this way, magnetically-responsive hydrogels with anisotropic fibrillar-like architectures have been proposed as biomimetic scaffolds for engineering and regenerating a wide range of damaged tissues such as cartilage,^[106] tendon,^[111] or cardiac tissues.^[109] Interestingly, MNPs can also be functionalized with reactive motifs to act simultaneously as crosslinking agents and structural nanoelements for the generation of fibrillar-like anisotropic microstructures within hydrogels without requiring additional compounds or stimuli.^[112]

The weak diamagnetic response of different fillers such as carbon nanotubes^[113] or the above-discussed CNCs^[100] can also be exploited to control their arrangement within polymeric hydrogels through the application of high-intensity magnetic fields to create anisotropic fibrillar-like composites. However, the required high-strength magnetic fields may compromise the viability of this approach. In this sense, it is worth mentioning that the synthesis of highly responsive MNPs allows the fabrication of magnetic fibrillar-like hydrogels by incorporating low amounts of MNPs and applying low-intensity magnetic fields, thus minimizing associated potential toxicity concerns.^[114] For a more in-depth discussion about the biocompatibility of MNPs depending on their concentration in biomaterials and their inherent physicochemical properties, we refer the readers to recent review articles that cover this topic.^[104,115]

3.2. Micro-Building Blocks and Microfillers

3.2.1. Polymeric Rod-Shaped Microgels

The use of anisometric polymer microgels as building blocks allows the fabrication of granular hydrogels with fibrillar-like structures that display controlled anisotropy, porosity, and/or structural guidance with potential applications in TE.^[116] Different fabrication routes can be followed for the synthesis of elongated microgels, including mechanical fragmentation,^[117] batch emulsion,^[118] microfluidic^[119] and lithography-based techniques.^[120] The resulting microgels can then be annealed into hierarchical granular hydrogels by several strategies, namely chemical crosslinking^[121] (e.g., enzymatic catalysis, radical polymerization) and physical forces^[122] (e.g., electrostatic interactions, hydrogen bonding). For example, the packing of rod-shaped hyaluronic acid (HA) microgels resulted in granular hydrogels with anisotropic porosity.^[116] In another approach, annealed strand-shaped microgels were used to form porous fibrillar-like hydrogels that support cell growth and proliferation.^[123]

Besides their controlled assembly to obtain annealed macroscopic structures, anisotropic microgels have been also proposed as potential microfillers of other hydrogel matrices. However, the

typically low aspect ratios of this type of microstructure limit their potential to generate fibrillar-like architectures within bulk hydrogels when used as simple microfillers. Interestingly, the modification of rod-shaped microgels with MNPs prior to their incorporation into hydrogels allows their subsequent remote magnetic arrangement. This strategy can be exploited to align the incorporated microgels, creating highly anisotropic fibrillar-like architectures. In a representative application of this approach, magnetically responsive rod-shaped microgels composed of poly(ethylene oxide-*stat*-propylene oxide) and iron oxide MNPs were first produced through a mold lithography-based technique and then crosslinked under an applied external magnetic field to obtain anisotropic fibrillar-like hydrogels (Figure 4a).^[120,223] On the other hand, polymeric microgels can also be formulated to incorporate signaling factors within hydrogels, such as PDGF-BB that induce the migration of encapsulated tendon progenitor cells,^[124] while providing them with a fibrillar architecture.

3.2.2. Electrospun Microfibers

Electrospinning is a versatile fabrication method to produce continuous polymeric fibers with diameters ranging from nano- to micro-scale (see Section 4.2 for more details). Electrospun microfibers can be sectioned and used as microfillers to generate hydrogels with fibrillar architectures. The electrospun meshes can be fragmented through chemical reactions,^[125] or mechanically sectioned to obtain fiber segments with lengths in the micrometer range. In this case, different approaches such as sonication,^[126] the repetitive passing of the fibers through a syringe needle upon hydration^[127] or their cutting in a microtome after embedding in a solidified hydrogel have been explored.^[128] After that, the incorporation of short microfibers can be exploited to physically remodel the micro- and macro-scale properties of soft cell-laden hydrogel environments, providing them with a desired fibrillar-like structure (Figure 4b). In this way, isotropic hydrogels loaded with short electrospun microfibers have been used to explore cell mechanosensing in 3D fibrillar environments,^[129] or towards tissue regeneration applications.^[124] Importantly, the stiffness of segmented electrospun microfibers has a remarkable impact on the biological performance of fibrillar hydrogels. For instance, microfibers with lower stiffness permitted active cellular forces to recruit nearby fibers within methacrylated dextran hydrogels, thus increasing ligand density at the cell surface and promoting the formation of focal adhesions and related signaling, while stiffer fibers limited cell spreading and proliferation.^[130] Importantly, the porosity of hydrogels created with microfiber segments can be controlled with fiber concentration, which is another key design factor in controlling the migration, morphology, and phenotypic commitment of the encapsulated cells.^[114]

Interestingly, electrospun microfiber segments can be aligned within the hydrogels rendering fibrillar-like composites with highly anisotropic architectures. Although the extrusion of hydrogel bioinks loaded with short microfibers during 3D printing processes has been effective for this purpose,^[127] the most common approach to orientate the microfillers is with MNPs. For instance, anisotropic fibrillar alginate-based hydrogels were designed by aligning the incorporated short PCL-MNP

electrospun microfibers under the application of an external magnetostatic field.^[131] Moreover, the sectioned microfibers used to generate biomimetic fibrillar networks within the hydrogels can also be leveraged to incorporate the desired biochemical cues in the systems. In a representative example of this strategy for tendon TE, cryosectioned electrospun microfibers composed of PCL, tendon dECM, and magnetite MNPs were magnetically aligned within GelMA hydrogels to enhance the tenogenic commitment of the encapsulated stem cells and the deposition of a tendon-like matrix.^[114]

Fragmented electrospun microfibers can be also annealed to form fibrillar hydrogels by themselves. To achieve this, norbornene-modified microfiber suspensions were assembled and crosslinked into a highly porous hydrogel with a fibrillar structure (Figure 4c).^[132] The presence of norbornene groups was exploited for the photocrosslinking of the microfibers, thus obtaining fibrillar scaffolds that allowed the encapsulation and spreading of human MSCs and programmable multicellular macroscopic contraction of the fiber assembly.

3.3. Feedback of Biomimetic Fibrillar Hydrogels on Cells Behavior

In our recent work, we showed the labeling and perturbation of early matrix interactions with ECM secreted by MSCs in 3D isotropic hydrogels, which altered their mechanosensing by transitioning interactions with ligands in the engineered microenvironment to those of their own deposited ECM.^[133] Such matrix labeling has also shown that cells deposit a matrix as they interact with fibrillar matrices, as observed with PCL-based fibers.^[134] It is still unclear how the secreted nascent cell matrix influences cell perception of soft hydrogel-based ECM fibers. In 2D studies with fibroblasts seeded on electrospun HA or dextran fibers, the presence of adhesion ligands was required for significant cell attachment, suggesting that these receptors are required for cells to sense the fibrillar topography.^[135,136]

Similarly, studies with fibroblasts encapsulated in the previously discussed short fiber-reinforced hydrogel composites, systems where electrospun microfiber fragments are encapsulated within a bulk isotropic hydrogel, showed that adhesion ligands are required for cells to engage with electrospun fibers.^[137] Although multiple studies have shown the deposition of cell-derived ECM (fibronectin, type I collagen) and associated plastic deformation of synthetic fibers, it is still unclear how a nascent matrix might assemble and alter cell mechanosensing in the presence of deformable soft hydrogel fibers.^[132,138] It is likely that mesenchymal cell-secreted matrix is involved in cellular sensing of nano- to micro-scale topographical cues imparted by fibers, that the composition of this matrix influences fiber engagement and force generation, and that nascent matrix needs to be remodeled for cells to sense fibrillar structures. Importantly, most work in this area has been carried out with mesenchymal or endothelial cells, and it is unclear how other cell types will respond to these microenvironments. Thus, the feedback of the fibrillar architecture of hydrogel scaffolds on cell response is a particularly unexplored research field that may lead to key discoveries for establishing the design rules of future TE concepts.

3.4. Incorporation of Biofunctional Nanoparticles

In most cases, fibrillar hydrogels obtained through the previously discussed fabrication strategies do not meet all of the characteristics required for their intended application. In the field of TE, for example, the design of scaffolds with fibrillar structures is only one of the requirements to be considered for the manufacture of hierarchical constructs that replicate features of native ECM. In this section, we briefly review the main NP-based modification strategies proposed to provide polymeric hydrogels with specific biofunctional properties, focusing on nanocomposite fibrillar hydrogels with increased electrical conductivity, remote magnetic responsivity, and enhanced mineralization (Figure 5).

3.4.1. Electrical Conductive Nanoparticles and Polymers

The incorporation of nanofillers with conductive properties within polymeric hydrogels is a well-explored strategy for the engineering of tissues that respond and communicate via electrical signals, such as cardiac, neural, or muscles (Figure 5a). For instance, the modification of alginate-collagen and GelMA hydrogels with carbon nanotubes has been demonstrated as an efficient design strategy to fabricate conductive constructs for cardiac TE purposes.^[139] Gold nanowires were incorporated in collagen-based hydrogels and aligned by the application of external electric fields before gelation, thus creating electrically responsive composites with anisotropic fibrillar-like structures.^[140] Other nanostructures such as silver NPs,^[141] or novel Ti₃C₂ MXenes nanosheets^[142] have been evaluated as potential options to enhance the conductive properties of polymeric hydrogels.

Besides the aforementioned carbon-based or inorganic NPs, polymers with inherent conductive properties have been also proposed not only as functional fillers to design electric responsive/conductive hydrogels but also to form fibrillar-like conductive hydrogel networks by themselves or in combination with other polymers.^[143] In a representative example of the first approach, polyaniline nanofibers were incorporated into collagen hydrogels for neural and muscle TE applications.^[144] On the other hand, it has been demonstrated that conductive hydrogels can be obtained through the in situ formation and interwoven of polydopamine-doped polypyrrole nanofibrils.^[145]

3.4.2. Magnetic Nanoparticles as Ordering and Remote Stimulation Tools

As discussed above, the incorporation of MNPs within polymeric hydrogels has been widely evaluated to generate constructs with anisotropic fibrillar-like architectures through the application of external magnetic fields. In the most typical approach, MNPs display a superparamagnetic behavior,^[146,147] thus precluding their agglomeration and improving control over their distribution/orientation. However, MNPs with a ferromagnetic response (i.e., that retain a certain magnetization after removing the applied magnetic field)^[148,149] have also been proposed in this field.

In addition to their use as structural agents, the presence of magnetic materials can also be exploited for the programmable remote stimulation of the hydrogel-based constructs

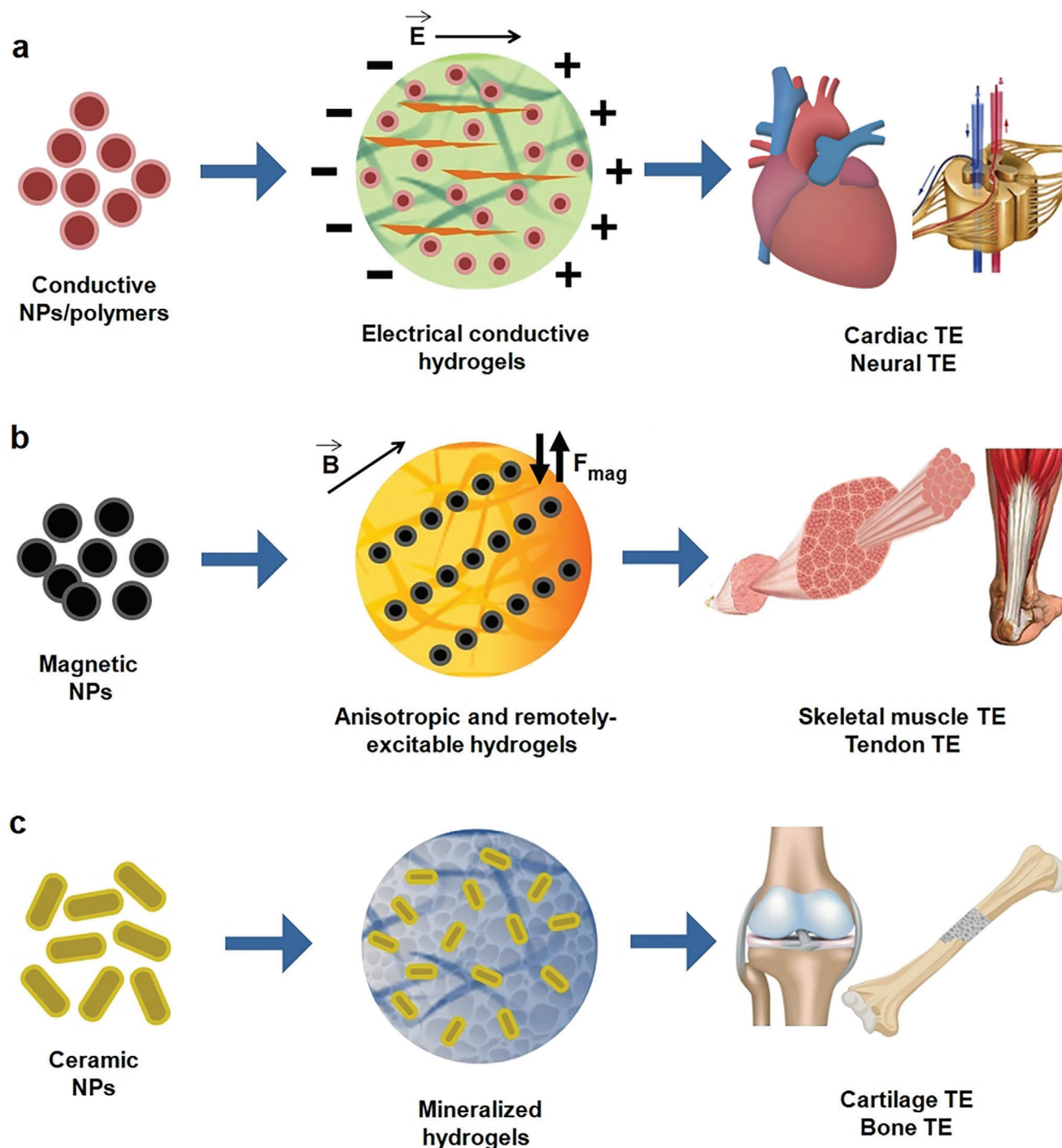


Figure 5. Nanoparticles provide fibrillar hydrogels with additional biofunctionalities. a) The incorporation of conductive NPs or polymers in hydrogel formulations can be exploited to provide them with the electrical behavior required to design biomimetic cardiac or neural tissues. Cardiac tissue drawing reproduced with permission.^[215] Copyright 2021, MDPI, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>); neural tissue drawing reproduced with permission.^[216] Copyright 2020, Elsevier, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>). b) The addition of magnetically-responsive nanostructures allows the generation of uniaxial anisotropic architectures within hydrogels and the magneto-mechanical stimulation of the encapsulated cells during maturation through the application of external magnetic fields. Skeletal muscle drawing reproduced with permission.^[217] Copyright 2017, Wiley-VCH; tendon drawing reproduced with permission.^[218] Copyright 2014, Danish Medical Association, CC BY-NC 4.0 (<https://creativecommons.org/licenses/by-nc/4.0/>). c) Ceramic NPs can be used to enhance the mineralization degree of polymeric fibrillar hydrogels in the engineering of load-bearing tissues. Bone drawing reproduced with permission.^[219] Copyright 2021, Elsevier; cartilage drawing reproduced with permission.^[220] Copyright 2021, MDPI, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

(Figure 5b).^[150,151] This feature is especially relevant in TE applications since it is well-known that magneto-mechanical stimuli play a key role in the regulation of cell behavior within hydrogel matrices.^[104,150–152] Conceptually, the application of external magnetic fields can be used to induce small movements of the incorporated MNPs, thus creating dynamic environments that more precisely replicate biological conditions. For instance, the application of oscillating magnetic fields during the in vitro maturation of cell-laden GelMA hydrogels modified with magnetically responsive microfibers activated cellular mechanotransduction pathways and increased the expression of tenogenic markers.^[114] Beyond exploiting their remote responsive properties for structural and stimulation purposes, the incorporation of MNPs has been also evaluated to provide hydrogels with other functionalities, such as hyperthermia and magnetic resonance imaging capabilities.^[153]

3.4.3. Ceramic Nanoparticles to Induce Mineralization

The modification of fibrillar hydrogels with ceramic NPs has been widely evaluated in the engineering of load-bearing tissues, not only to increase the strength and stiffness of the composites but also to enhance their mineralization (Figure 5c). Among all of the nanomaterials explored for this purpose, hydroxyapatite NPs are undoubtedly the most investigated fillers in the development of biomimetic mineralized hydrogels. It has been demonstrated that calcium and phosphate ions released after hydroxyapatite NP degradation within cell-laden hydrogels enhanced the deposition of mineralized matrix by osteoblasts without compromising cell viability.^[154] Moreover, in the specific case of bone TE applications, the incorporation of hydroxyapatite in the hydrogels also contributes to better mimicking the native tissue, since around 60–70% of bone ECM consists of inorganic hydroxyapatite nanocrystals.^[155]

The incorporation of hydroxyapatite NPs has been combined with different design approaches to fabricate mineralized hydrogels while generating a desired fibrillar-like structure. These include, for example, the addition of ceramic nanostructures in collagen hydrogels with inherent fibrillar structure,^[156] or in nanofiber-reinforced chitosan-based hydrogels.^[157] On the other hand, the design and incorporation of hydroxyapatite NPs with elongated morphologies can be exploited to simultaneously provide isotropic hydrogel matrices with a pseudo-fibrillar structure and enhanced mineralization properties.^[158]

The modification of polymeric hydrogels with other ceramic nanostructures such as Laponite XLG nanosilicates,^[159] nanoclays,^[160] or strontium-carbonate NPs^[161] have also been used to enhance their mineralization potential.

4. Manufacturing Technologies to Produce Fibrillar Hydrogels

4.1. Microfluidic Spinning

Microfluidic fabrication technologies enable the production of hydrogel microfibers with highly controlled architectures and cellular organizations (Figure 6a). The flows generated in the

channels of microfluidic devices display laminar behavior, which prevents mixing across the adjacent streams unless it is otherwise induced. This feature enables the processing of mono- or multi-component microfibers by inducing the immediate solidification of solutions upon extrusion with relevant crosslinking agents or triggers.^[162] Different materials, such as poly(lactic-co-glycolic acid) (PLGA),^[163] GelMA,^[164] or silk^[165] have been processed into fibers using microfluidic spun-based techniques. Depending on the chemical nature of precursor solutions, spun microfibers can be further stabilized by thermal-, ionic-crosslinking, or photopolymerization, among other methods.

In microfluidics, viscosity and surface tension determine the flow characteristics at interfaces between solutions within the microchannels. For example, using feed solutions with similar viscosities provides a stabilized laminar flow within the microfluidic devices and enables the continuous fabrication of straight fibers with uniform dimensions,^[166] whereas fluids with large viscosity differences (inner > outer) with a constant core and varying sheath velocity result in coiled microfibers.^[167] Moreover, in microfluidic systems with laminar flows, the diameter of the microfibers can be adjusted by changing the flow rates of core and sheath hydrogels.^[168] Besides viscosity and flow rates, modulating microchannel geometry, fluid concentrations, and/or gelation methods allows the fabrication of hydrogel fibers with different structures and diameters ranging from nanometers to a few hundred micrometers.^[169]

The simultaneous extrusion of multiple hydrogels using coaxial flow microfluidic devices has been widely explored to engineer multilayered microfibers that more closely recapitulate the complex topology of native tissues. For example, the three-layer coaxial laminar flow created in a microfluidic device was used to design core-shell microfibers loaded with different types of cells for neural TE purposes.^[170] Coaxial flows can also be exploited to control the folding of spun microfibers and generate helical alginate-based structures with potential application in blood vessel TE.^[171] On the other hand, to allow more precise control over the dimensions of microfibers (diameters down to $\approx 1.5 \mu\text{m}$), direct laser writing methods have been integrated with the design of microfluidic devices.^[172]

The flexibility of microfluidic spinning technologies allows the generation of microfibers with controlled surface chemistry and topography that can be further assembled into hierarchical 3D structures. For instance, endothelial cell-laden GelMA microfibers with biomimetic surface patterns were produced using devices with grooved channels.^[169] The integration of a static mixer into coaxial microfluidic devices has been suggested as an alternative strategy to produce hydrogel fibers with topographical cues. This component allowed the splitting of flows into sub-streams with predetermined sizes to induce the formation of internal filament-like microstructures within spun hydrogel fibers.^[173] Microfluidic technologies can also be exploited for the generation of accurate in vitro models that allow a better understanding of tissue disease progression and the development of adequate therapeutic approaches. In a representative example, a mimetic tumor microenvironment based on peptide-modified alginate fibers was fabricated using microfluidic concentric flow spinning methods.^[174]

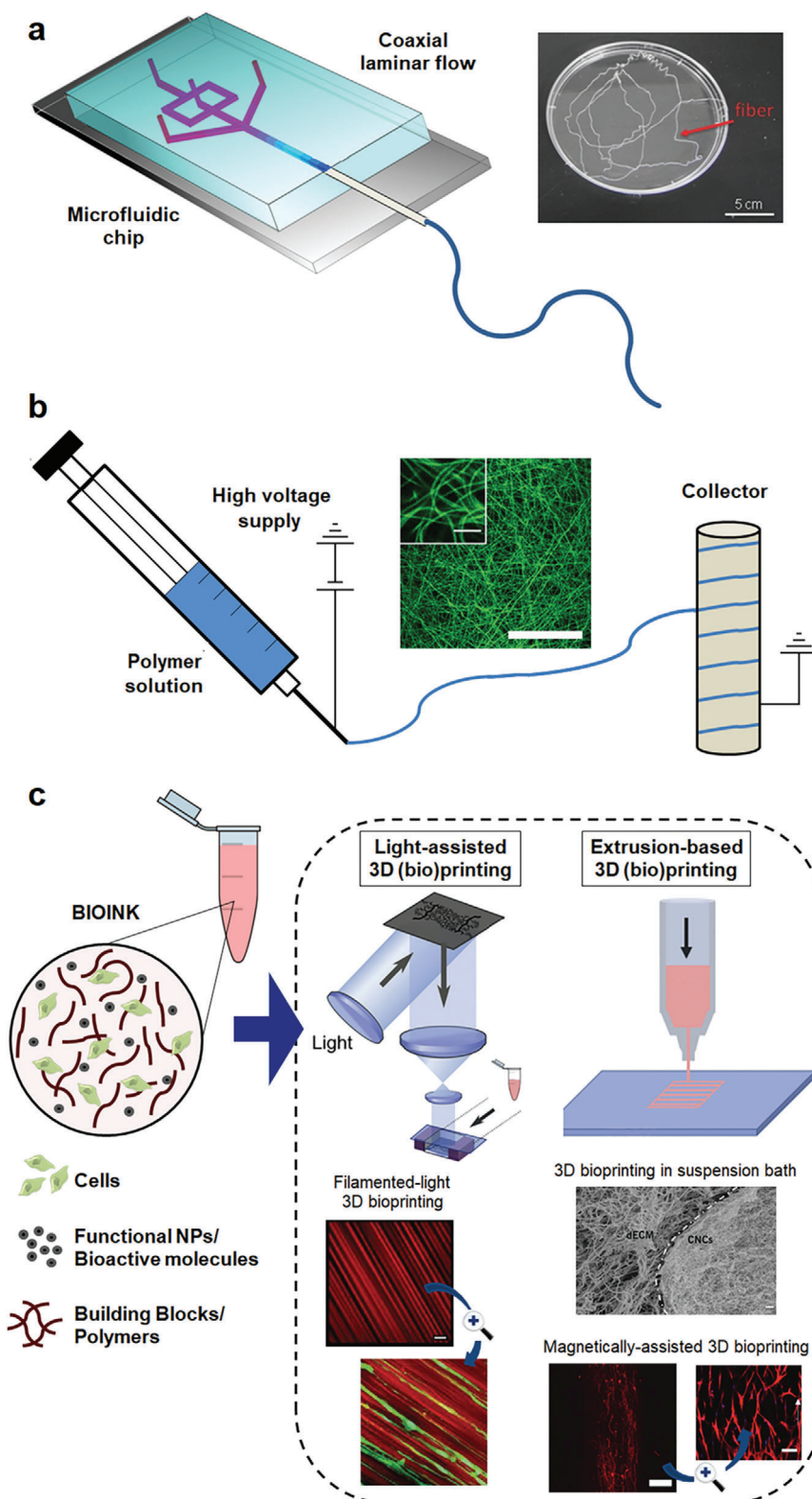


Figure 6. Fabrication technologies. a) Schematic illustration of microfluidic spinning fabrication technique. Photograph of GelMA-based microstructured fibers synthesized using this approach. Reproduced with permission.^[169] Copyright 2015, Wiley-VCH. b) Schematic illustration of electrospinning fabrication technique. Fluorescent microscopy image of electrospun HA-based microfibers. Reproduced with permission.^[184] Copyright 2019, American Chemical Society. c) Bioinks composed of a mix of cells, bioactive molecules, functional nanofillers, and the previously discussed polymers/building blocks can be processed through different 3D bioprinting approaches, with extrusion-based and light-assisted the most widely explored

4.2. Electrospinning

Electrospinning is a processing technique to obtain high-density polymeric fibers with a unique range of fiber diameters (≈ 50 – 5000 nm). This method is based on the extrusion of polymer solutions into electric fields that destabilize the surface tension of the polymer droplet, drawing out fibers that are dried in the air as they deposit on a charged collector (Figure 6b). Electrospinning has received attention from the bioengineering field due to the similarity of structure and size of the obtained fibers with components of natural ECMs, including on the same scale as collagen, fibrin, elastin, and fibronectin fibrils and fibers. It has been challenging to create fibers in this range with other methods, whereas fibers greater than microns are often produced with alternate techniques and synthetic self-assembled fiber gels exhibit fibers at the nano-scale.^[175]

Despite the detailed advantages, a key concern associated with electrospinning processing is the low solubility of the most typically used polymers in aqueous media. For example, the use of highly toxic organic solvents such as dichloromethane (DCM), hexafluoroisopropanol (HFIP), or chloroform is required for the production of electrospun microfibers. For example, collagen, a widely used natural polymer for the fabrication of electrospun microfibers for TE purposes, is largely insoluble in other solvents and the common solvent for it is HFIP. Most of these solvents have low boiling points and high volatility, thus favoring their evaporation during the spinning process. However, low amounts of solvent retained in the microfibers could influence the biological function of the microfibers. For example, it has been shown that the choice of solvent used in the electrospinning of polyvinyl alcohol (PVA) microfibers is critical in cell layering, as residual solvent in the electrospun microfibers is detrimental to mouse fibroblasts.^[176]

To overcome these challenges, the use of aqueous-based solutions for electrospinning has been also proposed, but it is limited by the low number of water-soluble polymers and the need for crosslinking/stabilization to limit dissolution. For example, chitosan was successfully electrospun after rendering it soluble in aqueous media through an alkalization-carboxymethylation procedure.^[177] In another strategy, water-insoluble polystyrene (PS) fibers were electrospun from aqueous solutions using PVA as a template polymer that acted as a glue between PS particles.^[178] Stabilization is also needed with electrospun scaffolds from natural materials to maintain their fibrillar structure and to improve mechanical properties. This crosslinking process may involve heat, such as dehydrothermal radiation, encompassing UV or γ -irradiation, or chemical agents.^[179] As a representative example, water-stable gelatin fibers can be obtained by post-crosslinking with genipin, a naturally occurring crosslinker with low cytotoxicity.^[180] These concepts could be extended to other types of polymers and natural molecules to expand the materials available for scaffold fabrication.

While most studies with electrospun fibers have utilized non-hydrogel polymers (e.g., thermoplastics), the number of studies that explored the design of hydrogel fibers through electrospinning techniques is still limited. Initial work in this area was focused on the processing of common synthetic hydrogel materials such as polyacrylamide and PEG, as well as natural materials such as HA and collagen, but this has expanded to many proteins (e.g., gelatin, keratin, elastin, fibrinogen, and silk fibroin) and polysaccharide (e.g., chitosan, dextran, alginate, cellulose, and pectin) materials, as outlined in comprehensive reviews.^[181,182] To mimic the mechanical and biochemical properties of fibrillar ECMs, the use of photochemistry has been indispensable, as it allows the easy tuning of hydrogel fiber mechanical properties and biochemical ligands. An important distinction here is that hydrogel fiber stiffness can be tuned to have bending moduli similar to natural ECMs,^[130] while it is unclear if thermoplastic fibers can be made this soft as their mechanical properties tend to be orders of magnitude higher than hydrogels.

The importance of fiber stiffness has been explored using many highly tunable hydrogel fiber systems. Initial work with methacrylated HA hydrogel fibers deposited on glass coverslips showed that soft fibers with lower amounts of adhesion ligands increased the chondrogenic potential of human mesenchymal stromal cells (MSCs), relative to stiff fibers with higher amounts of adhesion ligands.^[183] As discussed above, electrospun materials (e.g., dextran) have been used to assess the influence of fiber stiffness on fiber recruitment and mechanosensing.^[130] This finding is contrary to the case for isotropic hydrogels, where stiff substrates promote the spreading of MSCs, which highlights the importance of ECM structure and dynamics when studying mechanobiological pathways.

This suspended fiber system was then used to study the impact of fiber mechanics on endothelial network formation,^[138] hepatic and lung myofibroblast activation,^[136,184] or cell migration.^[185] Swelling-induced changes in fiber structure have also been explored as a means to create collagen-like crimped hydrogel fiber networks that have non-linear mechanical properties.^[186] Photocrosslinkable chemistries were utilized throughout all of these studies due to the high level of control over fiber formation and mechanical properties with light. This work shows that subcellular scale fibers obtained through electrospinning provide ECM-relevant structures that cells can reorganize similar to natural fibrillar ECM. This level of remodeling and rapid cell-mediated structural change has only been observed with soft electrospun hydrogel fibers.

4.3. 3D Bioprinting

3D bioprinting technologies enable the precise positioning of multicomponent cell-laden materials to create complex

to obtain cell-laden fibrillar hydrogels with high resolution and shape-fidelity. Schematic illustrations of printing setups adapted with permission.^[191] Copyright 2018, Elsevier. Microscopy images of cell-laden hierarchical fibrillar hydrogels fabricated through filamented-light-assisted, extrusion embedded in CNCs-based suspension bath and magnetically-assisted extrusion 3D bioprinting. Reproduced with permission.^[209] Copyright 2022, Wiley-VCH, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>); reproduced with permission.^[196] Copyright 2023, American Chemical Society; reproduced with permission.^[114] Copyright 2022, Wiley-VCH.

and hierarchical biomimetic tissues with controlled chemical and mechanical gradients. The biological functionality of the manufactured constructs is mainly dictated by the selected printing method and the properties of the printable living materials (bioinks).^[187] According to the standard document ISO/ASTM 52900:2015-12 Standard Terminology for Additive Manufacturing, 3D bioprinting technologies are classified into three main categories: biomaterial extrusion, biomaterial jetting, and vat photopolymerization.^[188] Although all of these technologies have been widely proposed for biomaterials manufacturing, the processing of hydrogel bioinks into 3D constructs through extrusion-based and light-assisted (e.g., vat polymerization) 3D printing systems are the most explored options in the biomedical field (Figure 6c). An ideal bioink for TE purposes should possess target tissue-specific chemical and biological properties, printability (bioprinter-compatible rheological properties and gelation mechanisms), high biocompatibility, and adequate mechanical strength, among other requirements.^[189] Due to their high cytocompatibility and easy gelation, certain materials such as gelatin, alginate, or agarose have been the most widely proposed as hydrogel bioinks.^[190,191]

Although many advances in hydrogel fiber fabrication have been described, very few studies have combined these technologies with additive manufacturing techniques. This is likely due to stringent requirements for extrusion bioinks and also for photocrosslinkable systems utilized in light-based techniques. For example, extrusion 3D printing of keratin fibers has been used to create shape memory microfibrillar scaffolds that can undergo programmed shape changes upon hydration.^[192] Electrospun fiber assemblies are extrudable and photocrosslinkable, which allows for the extrusion printing or photopatterning of cell-laden filaments with complex shapes that undergo cell-mediated compaction and bending.^[132] In another example, HA fibers combined with uncrosslinked GelMA macromers and cells were extruded causing the shear-induced alignment of the fibers to direct cell organization.^[127]

The combination of 3D printing with other technologies can be exploited to improve control over the microstructural characteristics of fabricated constructs. In a representative study, extrusion-based printing with multidispensing modules has been used to deliver multiple bioinks and print them together with a sacrificial mold to obtain muscle fiber-mimetic structures with $\approx 400\ \mu\text{m}$ in diameter.^[193] 3D printers can also be combined with microfluidic spinning systems and coaxial extruders to introduce alignment via multicellular fibrillar constructs.^[194]

Major challenges in current extrusion-based 3D printing systems are the poor manufacturability of soft hydrogel bioinks and the relatively low control over their spatial organization during the printing process. The use of viscous support printing baths that quickly recover after any mechanical disruption (nozzle movement) has emerged as a powerful fabrication strategy to overcome these limitations, allowing the high-resolution printing of complex filamentous structures. Aqueous^[195] and nanoparticle-based baths^[102,196] are particularly interesting options for this strategy, allowing the fabrication of constructs with high resolution and shape fidelity, as well as their immobilization into a hierarchical fibrillar environment to maintain long-term cell culture.

5. Translational Considerations

In this section, we briefly list some of the main remaining barriers to the effective clinical translation of engineered fibrillar hydrogels. There is a wide range of general concerns associated with the advance of regenerative medicine products from the laboratory to the clinic. Thus, it is worth mentioning the low cost-effectiveness of end products in highly personalized fabrication approaches (e.g., 3D bioprinted fibrillar hydrogels), the existing ethical considerations (e.g., source, donation, and manipulation of cells), or the different regulatory frameworks in each country (e.g., nation-specific authorization processes, requirements, and timings).^[197–199] However, focusing on the specific case of fibrillar hydrogels designed for TE applications, we identified three major areas that need future work to improve the clinical applicability of the engineered constructs.

- A) Improved understanding of tissue complexity and heterogeneity.^[103,200,201]
 - The complex hierarchical and anisotropic fibrillar structures of some tissues and interfaces have yet to be completely deciphered, including different preferential orientations and dimensions of fibers depending on tissue area/depth (e.g., cartilage, enthesis, muscle).
 - A better understanding of the matrisome and the hierarchical assembly of proteins and structure across individual tissues and between different tissues is needed.
 - Knowledge of gender or ethnic differences in tissue organization and mechanical properties is still limited and is essential to increase the success of equitable clinical therapies.
- B) Structural and biological limitations of current fabrication approaches.^[187,199,202]
 - Fibrillar hydrogels that allow user-controlled modulation of matrisome cues are still limited, which is required to recreate the ECM changes found during tissue development or pathological processes (e.g., fibrosis).
 - Meeting biofabrication requirements, such as printing windows that balance processing and resolution. Most of the above-discussed fibrillar hydrogels cannot be processed with both fidelity and cytocompatibility, delaying the clinical translation of personalized bioprinted constructs.
 - Potential cytotoxicity and/or low biodegradability of several of the building blocks/fillers used to fabricate fibrillar hydrogels (e.g., MNPs, PCL microfibers, cellulose NPs).
 - High risk of batch variability of natural components used to fabricate fibrillar constructs (e.g., collagen and laminin batches) and cell sources.
 - Poor revascularization rate and integration of complex hierarchical constructs with the host vasculature. This includes the ideal scale of fibrillar architectures to allow the invasion of scaffolds by native tissues.
- C) Main concerns associated with the scale-up of fibrillar hydrogels.^[202,203]
 - Recreating hierarchical complexity (both physical and chemical) and long-term fibrillar structures in human-sized engineered tissues/organs.
 - Development of effective and functional microvascular networks – poor vascularization in the center of large-sized fibrillar constructs.

Table 1. Main advantages and limitations of different materials proposed as (nano/micro) building blocks or fillers to fabricate fibrillar hydrogels.

Building block / filler	Materials	Advantages	Limitations	Key ref.
Natural fibrillar proteins	Collagen Fibrin Matrigel dECM	Retention of native tissue complexity. High cytocompatibility. Proteolytically degradable and amenable to cell-mediated remodeling. Capability of enzymatic degradation-mediated remodeling (e.g., pepsin).	Complex protein purification. High batch-to-batch variability. Xenogeneic origin. Fast proteolysis and low in vitro stability. Weak and difficult-to-modulate mechanical properties.	[41, 48, 54, 55, 61]
Block copolymer micelles	PEG- <i>b</i> -PHPMA ^{a)} PEO- <i>b</i> -PS ^{b)} PMAA- <i>b</i> -P(St- <i>alt</i> -NMI) ^{c)} PEO- <i>b</i> -PBO ^{d)}	Easy and tunable fabrication route. Typically reversible gelation (e.g., temperature).	Potential undesired kinetic effects. Limited physical/mechanical properties. Short-length micelles make it difficult to induce fibrillar structure. Low-size hydrogels for clinical application.	[75–78]
Peptides	Aromatic groups-modified peptides RADA 16-I Ovalbumin pept. Oligopeptides	Easy fabrication. Simple chemical and biological decoration.	Limited physical/mechanical properties. Typically low-size hydrogels for clinical application.	[80, 84, 85, 87, 89, 92]
Cellulose nanofibers and nanocrystals	(source) Wood products Bacterial walls Sea plants Tunicate	Biological origin. Easy functionalization (polymers, GFs, etc.) due to abundant surface groups. High mechanical properties.	Potential cytotoxicity. Difficult to establish standard protocols for fabrication due to different sources. Non-biodegradable in vivo.	[97, 100–102]
Magnetic nanoparticles	Iron oxide Doped ferrites Nickel	Remote arrangement to create fibrillar hydrogels with different anisotropic architectures. Remote non-invasive stimulation of cell-laden hydrogels during maturation.	Potential toxicity of magnetic materials. Safety risks associated with the application of magnetic fields. Potentially uncontrollable aggregation.	[104, 106, 107, 109, 110]
Rod-shaped microgels	HA ^{e)} HA + human platelet lysate Alginate Collagen	Easily tunable physicochemical properties (composition, shape, porosity). Potential magnetic modification and remote arrangement.	Difficulty in controlling assembly of microscale building blocks. Typically low aspect-ratio.	[116, 120, 121, 123, 221, 222]
Electrospun microfibers	PCL ^{f)} PLA ^{g)} PEG HA Polyacrylamide Collagen Chitosan	Controllable orientation to form anisotropic architectures. High and tunable aspect ratio resulting in highly fibrillar structures. Potential magnetic modification and remote arrangement.	Potential undesired biological effects associated with most typical materials (e.g., PCL). Potential undesired entanglement in anisotropic fibrillar hydrogels.	[114, 127, 128, 131, 132]

^{a)} PEG-*b*-PHPMA: poly(ethylene glycol-*b*-2-hydroxypropyl methacrylate); ^{b)} PEO-*b*-PS: poly(ethylene oxide-*b*-styrene); ^{c)} PMAA-*b*-P(St-*alt*-NMI): poly(methacrylic acid-*b*-styrene-*alt*-N-phenylmaleimide); ^{d)} PEO-*b*-PBO: poly(ethylene oxide-*b*-butylene oxide); ^{e)} HA: hyaluronic acid; ^{f)} PCL: polycaprolactone; ^{g)} PLA: polylactic acid.

- Achieving high degrees of nutrients and oxygen transport between thick tissue compartments.
- Retaining active growth factors within large constructs and precisely controlling their concentration and release over time throughout anisotropic biomimetic fibrillar networks.

6. Outlook

The easy tunability of physicochemical properties and suitability to encapsulate and direct cell fate make fibrillar hydrogels unique biomaterials for 3D cell culture, in vitro modeling, or TE applications. The design space available to produce hydrogels with biomimetic fibrillar architectures is wide and includes strategies such as the direct assembly and/or the use as fillers of different

structures ranging from proteins, peptides, cellulose NPs or spun microfibers to MNPs, among others (Table 1).

However, there are still many challenges related to the design and application of these systems in the biomedical field. The complexity of in vivo ECMs and cell-ECM interactions is the first aspect that hinders the re-creation of their biological and physicochemical properties in engineered scaffolds. Therefore, better understanding of the matrisome, that is, the genes encoding ECM and ECM-associated proteins, over different tissues and conditions, and the hierarchical assembly of proteins into tissues might allow us to better comprehend how single fibrous building blocks contribute to healthy and diseased states. Importantly, this might inform the design of new fibrillar and hierarchical biomaterials that influence cellular outcomes and tissue functions.

In terms of fabrication methods, the discussed strategies have achieved remarkable control over the hierarchical fibrillar structures and stiffnesses of hydrogel-based scaffolds. For instance, the complex highly anisotropic uniaxial architecture of tendons or the high elastic moduli of bones have been successfully reproduced in engineered hydrogels.^[114,204] Nevertheless, in most cases the designed fibrillar hydrogels do not replicate the presence and distribution of molecular components of the ECM, such as GAGs or proteoglycans. The incorporation of these molecular entities should be considered to faithfully replicate the composition of native tissues in future formulations. Existing developments being made in the fields of synthetic biology and biopolymer synthesis, and controlled self-assembly will certainly contribute to the bottom-up building of hierarchical fibrillar structures with readily tailored functionalities at the molecular level.^[205] The proper re-creation of other key mechanical properties of native ECMs, such as their viscoelastic response, and their impact on cell fate has not been fully accomplished yet. Importantly, fibrillar hydrogels typically display nonlinear stress-strain responses under tension due to reorientation and alignment of the fiber elements, positioning this class of biomaterials as advantageous platforms to better recreate the viscoelastic response of natural ECMs and living tissues. Additionally, because they enable changes to the bulk properties of the systems without changing the nanoscale elasticity of fiber structures that cells sense through focal adhesion, fibrillar hydrogels have unmatched potential when compared to other biomaterials for controlling both intrinsic mechanical properties and architectural features.

Reproducing the complex gradients in composition, structure, mechanical properties, and cell phenotype of most tissue interfaces on a single scaffold is still challenging. Since tissue interfaces are usually among the most prone to damage/injuries, increased effort should be directed toward the generation of fibrillar hydrogels with defined physicochemical gradients. For example, the use of novel and still unexplored dual nanofillers such as magnetoelectric^[206] or magnetoceramic^[207] NPs could be exploited to create hydrogel-based scaffolds with different anisotropies through the application of magnetic fields while providing them with conductive or mineralization properties, thus mimicking the transition between different regions of cardiac muscles and tendon-to-bone interfaces, respectively. Fiber spinning technologies such as microfluidics that enable the design of systems that selectively colocalize or compartmentalize gradients of multiple components in a single nano/microfiber are also promising for the fabrication of complex fibrillary hydrogel biomaterials.

Providing manufactured hydrogels with dynamic and stimuli-responsive properties is another factor that is sometimes not properly considered in the development of biomimetic scaffolding materials, although it is a key design feature to control the growth and differentiation of encapsulated cells. The stimulation of fabricated constructs through the application of invasive forces can compromise the integrity of the hydrogels.^[208] For this reason, the incorporation of magnetic-, electric- or light-responsive nanofillers has been explored to control the spatiotemporal changes of cell microenvironments, which can also elicit specific on-demand responses from encapsulated cells.^[187,205] However, the toxicity concerns associated with most of these nanomaterials can compromise their biomedical application.^[104] The

next generation of fibrillar hydrogels should consider the careful design of the incorporated stimuli-responsive nanofillers to allow the remote stimulation of the scaffolds by incorporating low amounts of inorganic materials and by applying weak stimuli, thus minimizing the potential toxicity issues of this strategy.^[104] Such materials could create new highly-cytocompatible soft biological robotics that can be safely actuated through external forces to control cell organization and fate.

In terms of manufacturing technologies, 3D bioprinting systems are undoubtedly among the most powerful techniques for increasing the level of control over the hierarchical complexity of fibrillar hydrogel constructs. However, as a recent and fast-paced field in continuous development, further advancements (e.g., hardware and software, materials science) are still needed to reach the promise of the field. One example of these innovative systems is the recently proposed filamented light (FLight) biofabrication technology, where an incident light radiation is optically divided into filamented beams that locally polymerize a photoactive hydrogel bioink, allowing the rapid and precise fabrication of cell-laden constructs composed of highly aligned unidirectional microfilament networks.^[209]

These few unexplored design/fabrication approaches identified here demonstrate that this field is still far from being fully explored. We hope that this review will foster advances that will be the basis for the fabrication of the next generation of biomimetic fibrillar hydrogel-based scaffolds, thus helping to bridge the existing gap between laboratory and clinical practice discussed in the previous section of translational considerations.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript. Researching data for article (A.P., M.G.F., M.D.D., and M.O.O.O.), discussion of content (A.P., M.G.F., R.M.A.D., J.A.B., and M.E.G.), writing (A.P., M.G.F., M.D.D., and M.O.O.O.); review/editing of manuscript before submission (A.P., M.G.F., R.M.A.D., J.A.B., and M.E.G.).

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Alberto Pardo completed his Ph.D. in materials science at the University of Santiago de Compostela (USC) in 2019. After holding a postdoctoral position at I3Bs - Research Institute for Biomaterials, Biodegradables and Biomimetics (University of Minho, Portugal) for 4 years, he returned to USC in 2023. Currently, his major research interest covers the design of advanced magnetic nanomaterials and their application in the fields of tissue engineering and cancer therapy and diagnosis.



Manuel Gomez-Florit is an associate researcher at the Health Research Institute of the Balearic Islands (IdISBa), Spain. His research interests include the development of advanced biomaterials and therapies for tissue engineering and regenerative medicine for the musculoskeletal system.



Rui M. A. Domingues is a senior researcher at I3Bs – Research Institute for Biomaterials, Biodegradables and Biomimetics, University of Minho, Portugal. He received his Ph.D. in chemical engineering from the University of Aveiro. His research focuses on the development of functional biomaterials and biofabrication concepts for tissue engineering and regenerative medicine applications. He has particular interest on nanostructured and nanocomposite biomaterials with biomimetic features and how they can be explored to control cell fate in tissue-engineered systems.



Jason A. Burdick is the Bowman-endowed professor in the BioFrontiers Institute and Department of Chemical and Biological Engineering at the University of Colorado, Boulder. His group designs new biomaterials and biofabrication methods for the development of translational therapeutics and tissue models for musculoskeletal and cardiovascular applications.



Manuela E. Gomes is an associate professor with Habilitation and Vice-President of the I3Bs Research Institute of the University of Minho, Portugal. Her research interests focus on tendon tissue engineering strategies, namely in the development of magnetic scaffold materials and hydrogels/bioinks based on biodegradable natural origin polymers, stem cell sourcing, and differentiation.