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Minimalist Tissue Engineering Approaches Using Low Material-Based Bioengineered Systems

Clara R. Correia, Isabel M. Bjørge, Sara Nadine, and João F. Mano**

From an "over-engineering" era in which biomaterials played a central role, now it is observed to the emergence of "developmental" tissue engineering (TE) strategies which rely on an integrative cell-material perspective that paves the way for cell self-organization. The current challenge is to engineer the microenvironment without hampering the spontaneous collective arrangement ability of cells, while simultaneously providing biochemical, geometrical, and biophysical cues that positively influence tissue healing. These efforts have resulted in the development of low-material based TE strategies focused on minimizing the amount of biomaterial provided to the living key players of the regenerative process. Through a "minimalist-engineering" approach, the main idea is to fine-tune the spatial balance occupied by the inanimate region of the regenerative niche toward maximum actuation of the key living components during the healing process.

1. Introduction

Despite the tremendous progress of tissue engineering and regenerative medicine (TERM) strategies in integrating cell biology principles with material science, very few have been translated to the clinic. A reasonable explanation is the lack of satisfactory biomorphological and biofunctional features of the tissue engineered constructs. While at the beginning of the TERM field it was believed that engineered structures should replicate the architecture of the tissue microenvironment from the microscopic to macroscopic dimension levels, nowadays it is well-established that there are other and more relevant key criteria that should be integrated during the design of biomaterials.[1–3] This paradigm shift in the TERM field was boosted by a deepened knowledge about the complex endogenous tissue repair process, which contributed to the identification of the key players involved, including cells and signaling molecules, and how the orchestration of spatial and temporal cues occurs *in vivo*.[4,5] Since then, a wide range of bioengineered strategies with tunable biophysical and biochemical characteristics have been proposed. These strategies aim to guide the bioperformance of endogenous progenitor or stromal/stem cells that are recruited to the injury site. In fact, from an "over-engineering" era in which biomaterials played a central role, we now witness the emergence of "developmental" tissue engineering (TE) strategies. "Developmental" strategies also rely on an engineering-based narrative, where an integrative cell-material approach paves the way for cells to self-organize, while underlining the importance of the extracellular matrix (ECM) and cell–cell contacts toward tissue reconstruction. In this report, we start by acknowledging the power of self-organization during tissue healing and how such ability should not be neglected in the design of tissue engineering strategies aiming toward tissue healing. Such principles have been explored in particular scaffold-free approaches,[6–8] another rapidly progressing field where biomaterials are completely absent, thus avoiding any risk of contamination from degradation products and inflammatory response resulting from the materials. For that, a series of signals are provided, including mechanical, chemical, topographic and geometrical guidance. In this review, we highlight TERM strategies that aim to establish an appropriate biomaterial-cell content balance, i.e., strategies that aim to fine-tune the balance inanimate engineered region, composed by implanted biomaterials, with the living region, composed by recruited cells and their essential counterparts, such as the newly deposited ECM. Such strategies are herein termed as low-material based TE strategies. Our main message herein assets in the minimization of the quantity of implanted biomaterials, as opposed to an excessive in situ overloading that ultimately leads to the failure of the TE strategy. Of note, we are aware of the great challenge in defining such minimal amount, and thus low-material based approaches should be carefully analyzed and take into consideration tissue- and biomaterial-specific factors, ranging from the physicochemical characteristics of the damaged tissue, mechanical loading, resident cell characteristics, namely proliferation ratio and density, biodegradation, as well as the extent of the lesion. Upon implantation, the "minimalist material engineering" approaches herein proposed will greatly benefit if designed to work in tandem with the recruited cells, including endogenous progenitor or stromal/stem and immune cells. For that, low-material based bioengineered systems can express at their surface biochemical cues able to facilitate or suppress cellular reprogramming toward a specific differentiation route, and thus, matching the type of tissue to be

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regenerated. For that, different low-material based strategies are herein highlighted. Such strategies are divided according to their complexity and architectural features, namely in *i)* cell and spheroid engineering as 0D cell-only systems, *ii)* cell sheets and thin films as one-dimensional (1D) systems either composed by cells, biomaterials, or a combination thereof, *iii)* microparticles and individual short-fibers, mainly applied as bottom-up structures providing cell adhesion sites and allowing cells to self-organize into highly hierarchical structures, and ultimately *iv)* macrostructures, which are further divided into ultrathin bulk scaffolds, liquefied systems, template-based systems, and hybrid three-dimensional (3D) bioprinted systems. Of note, such structures are not exclusively low-material based strategies, since most of them are classical examples widely used in tissue engineering. Therefore, the concept of "minimalist material engineering" is also dependent on how such systems are applied. It is thus our intention to tackle the TERM community to fine-tune the use of the available technologies to construct bioengineered systems, while recognizing the inherent ability of cells to self-organize. The main challenge is to design strategies requiring minimal *in vitro* manipulation but with maximum regeneration potential.

2. Stem/Stromal Cells Self-Organization Toward Tissue Development

Currently it is known that both embryonic and adult tissues with different cell types, architectures, and functions, are formed by self-organization of homogenous population of stromal/stem cells. The basic process of self-organization, which overlaps in time and space, can be obtained by *i)* self-assembly, a time-controlled process characterized by the rearrangement of cell positions, *ii)* self-patterning, a spatiotemporal event where a homogeneous population of cells acquires heterogeneous features, and *iii)* self-driven morphogenesis, to achieve tissue intrinsic shape in response to spatial and temporal cues.[9] Outstanding progress has been obtained in cell morphogenesis through the *in vitro* 3D culture of embryonic stromal/stem cells (ESCs), induced pluripotent stromal/stem cells (iPSCs), and tissue-specific adult stromal/stem cells. These 3D structures require an appropriate cascade of biochemical events to positively influence self-organization. For example, the spontaneous generation of ESCs into hindbrain neural-tube-like neuroepithelium structures requires fibroblast growth factor 19 (FGF-19) with a sequential addition of stromal cell-derived factor 1 (SDF-1) supplementation.[10] Moreover, the rearrangement of multicellular assemblies are biomechanically regulated by contractions between neighboring cells.[11] Both the ECM and cell–cell contacts were shown to be imperative to direct stromal/stem cell organogenesis.[12,13] The use of both natural and synthetic hydrogels acting as ECM has been successfully proposed for the self-generation of different types of organoids.[14–16]

In the last decade, different specific structures that resemble the *in vivo* architecture of tissues have been developed *in vitro*, including cerebral organoids,[17] optical-cup structures,[18] or even the epithelial structure of the gastrointestinal wall.[19] Moreover, the resulting organoids were shown to be an elegant idea for regenerative medicine applications. The transplantation of small colon organoids obtained through leucine-rich repeat-containing G-protein coupled receptor 5 ($Lgr5^{+}$) endoderm cells, improved the repair of the colon epithelium by adhering and covering the superficial damaged tissue.[20] Besides, heterotypic cell mixtures have been shown to self-organize into functional vascularized organ buds.[21] Considering that one of the fundamental TE challenges is the tissue microvasculature supply, such strategies are ideal to provide essential molecules and oxygen for cell survival in the functional developed tissues. However, only few types of cells possess the inherent ability to achieve this complexity stage when provided with relatively minimal biochemical inputs, and most of these organoid 3D cultures present key differences from the native tissues. In fact, even organoids are often limited to the millimetric scale, and thus far beyond the complex architectural macrofeatures of native organs. Therefore, the combination of organoids with biomaterials and fine-tuned engineering technologies, such as bioprinting,[22] can provide a better control over tissue size and architecture to ultimately develop artificial organs for regenerative medicine or even organ replacement.

3. The Native Microenvironment During Tissue Regeneration

During tissue regeneration, a set of events resembling morphogenesis begins to develop ordered multicellular functional structures. The tissue regeneration process involves a cascade of coordinated events, simultaneously with the secretion of various bioactive biochemical factors to promote cell recruitment.[23,24] Self-renewal activity during the regenerative process depends on the type of tissue or organ, and may occur rapidly, as is the case for blood and skin tissues, or be practically nonexistent, such as in the heart and central nervous system. Following tissue injury, the stromal/stem cell niche provides a source of quiescent cells that migrate into the site of inflammation to restore normal tissue homeostasis. Stromal/stem cell niches are available in many adult tissues such as bone marrow,[25] heart,[26] skin,[27] among others, and are a source of different endogenous stromal/stem cells that can control their renewal as well as their progeny. The synergic effect between recruited stromal/stem cells, immune cells, and resident differentiated tissue cells, dictates the success or the failure of the regeneration process. Innate immune cells are the first players to arrive at the injury site and play a central role in determining the quality of the repair response, including the extent of wounding, and the precision to restore tissue and organ functions. Furthermore, they maintain tissue homeostasis by engulfing cellular and matrix debris, remodeling the ECM, and releasing inflammatory and chemotactic mediators.[28] Recruited macrophages act simultaneously with tissue-resident macrophages and undergo marked phenotypic and functional changes in response to the tissue microenvironment.[29] Impaired interactions between macrophages and the tissue microenvironment can lead to aberrant tissue repair and may contribute to the loss of the regeneration ability in later stages of development.[28] Moreover, it is imperative that a high number of endogenous stromal/stem cells and progenitor cells migrate through the ECM from surrounding tissues to the repair site to subsequently undergo differentiation.

Additionally, the tissue regeneration microenvironment is full of heterogeneous cell types. The interaction between multiple cell phenotypes in cocultures has been shown to drive tissue

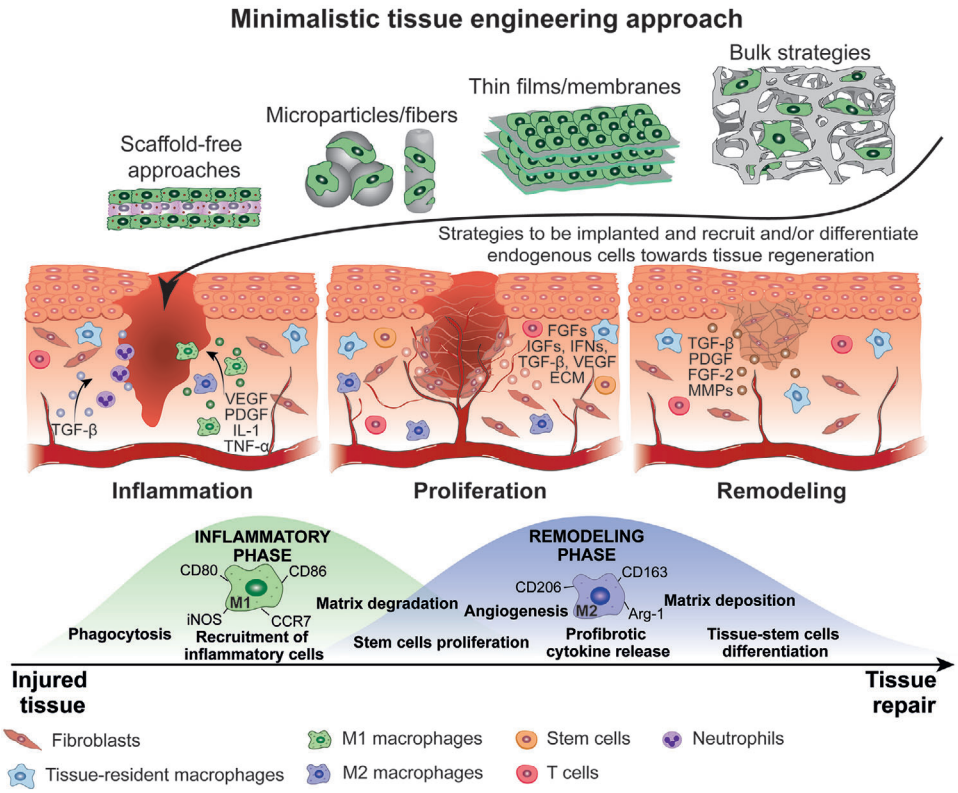


Figure 1. Schematic representation of the microenvironment of the regenerative niche. The orchestration of the cellular processes upon tissue damage is strictly dependent on the interaction of recruited endogenous cells with implanted bioengineered systems. In particular, the bioengineered systems represented are examples of low material-based tissue engineering strategies focused on minimizing the quantity of biomaterial provided to the living key players of the regenerative process in an attempt to do not jeopardize their innate self-organization ability.

formation and maintain the potency of stromal/stem cells during their expansion.[30] For instance, the direct contact between cardiosphere-derived cells and cardiomyocytes is fundamental for regeneration after myocardial infarction.[31] In bone, cell–cell contact between mesenchymal-derived and endothelial stromal/stem cells was shown to induce the generation of pericytes-like cells, critical to promote vascularization.[32]

The maintenance of tissue development, homeostasis, and repair is also controlled by a well-orchestrated array of secreted signals, including hormones, growth factors, and cytokines.[33] Usually, these bioactive signals are released by adherent cells, which mediate the communication between themselves and close neighboring cells. The secreted factors can drive cell stemness, differentiation, survival, and motility.[34,35] Furthermore, they play a key role in the formation of a mature vascular system.[36] Some examples include angiopoietins, bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), tumoral growth factors, vascular endothelia growth factor, among others well described in the literature.[33,35,37] A great strategy to control stromal/stem cell recruitment for tissue regeneration is the design of platforms for the controlled release of such biochemical signals.

Cells however are not the only providers of bioactive molecules to modulate stromal/stem cell differentiation, tissue repair, and regeneration. The products resultant from the degradation of the ECM have been shown to present chemoattractive and mitogenic properties on stromal/stem cells.[38–40] For instance, the *in vivo* administration of ECM degradation products into a digit defect site resulted in the recruitment of endogenous stromal/stem and progenitor cells with capability to differentiate into neuroectodermal and mesodermal lineages.[41]

Figure 1 schematically represents the orchestration of the tissue regeneration process events, while highlighting key biomolecules present in the regenerative niche. Additionally, examples of low-material based TE strategies, which are discussed in detail in Section 5, are also illustrated.

4. Engineered Biomaterials with Physical/Mechanical Bioactive Cues

Biomaterials providing physical, biochemical, and mechanical cues are put in play to control cellular organization or response down to the nanometric level. Mechanotransduction appears as a crucial mechanism where physical and mechanical cues from the native or engineered ECM are translated into biochemical signals.[42] Aspects such as substrate stiffness,[43] chemistry and exposure to bioactive factors,[44] or surface roughness,[45] viscosity,[46] topographical,[47] and geometrical[48] features affect cell adhesion and consequently activate specific downstream signaling pathways. Engineered microenvironments present the capability to not only mimic the native ECM but, more importantly, enable a control over cellular functions.

Cell adhesion to the ECM is mediated via transmembrane proteins, such as integrins, which bind to actin fibers via adaptor proteins. In response to binding, mechanical loading, and consequent force-induction is exerted on adhesion sites, which induce protein conformational changes and initiate downstream signaling.[49,50] Integrin-mediated adhesion triggers an elastic resistance toward deformation of the myosin contractility-powered continuous actin flow toward the center of the cell. Consequently, mechanical and physical properties of the substrate will lead to distinct resistance profiles and consequently force loading rates. Whereas rigid substrates produce higher force loading rates that induce unfolding of force-sensitive protein talin, this does not occur with lower force loading rates in response to soft substrates. Simplistically, talin unfolding exposes specific domains for vinculin attachment, which decreases actin flow, and enables growth of adhesion sites with consequent initiation of downstream signaling.[43,50] Increasing substrate rigidity is linked to an increase in actomyosin contractility, which has been correlated with lineage-specific differentiation. For instance, mesenchymal stem cell (MSC) differentiation is predominantly osteogenic on rigid hydrogel substrates, whereas intermediate rigidity induced myogenesis, and neurogenesis was promoted on soft substrates.[51] However, stress relaxation does play a crucial role independently from rigidity, particularly for cell encapsulation strategies. Viscoelastic faster relaxing hydrogels accelerate adhesion-ligand clustering and cell shape adjustment, promoting actomyosin contractility. Consequently, for an identical rigidity, encapsulated MSCs tend to differentiate toward the osteogenic lineage for faster relaxation rates, whereas adipogenesis is promoted for slow relaxation rates.[52] For purely viscous substrates with no elastic component, higher substrate viscosity has also been demonstrated to increase loading rates and actomyosin contractility, with promotion of myogenic differentiation.[46]

Physical cues, such as topographical or geometrical features, allow to finely tune cell spreading and morphology via confinement strategies.[53] These structures allow to control the size and shape of formed focal adhesions (FA), which have been shown to be dependent on actomyosin contractility and to impact consequent downstream signaling.[54] An interesting event for cells cultured on grooves is the occurrence of nuclear elongation, which impacts the repositioning of chromosomal territories, and can have potential implications on lineage-specific differentiation via modulation of transcription factors.[55] Of course, groove/ridge dimensions play a crucial role in the process and this has been specifically studied for osteogenic lineage commitment, where cell population was shown to be a key aspect. A common denominator however was the fact that maximum cell elongation, and by association greatest intracellular tension, was achieved for each maximum osteogenesis promoting dimensions.[56] In turn, promising results regarding upregulation of gene expression of neuronal markers were observed for neural progenitor cells cultured on nanogrooved and combination nanopit-microgrooved substrates, correlated with increased FA formation and higher actomyosin contractility.[54,57,58] Independently, the impact of nanopit depth on spreading and attachment of osteoblastic cells has been attributed to selective integrin subunit binding[59] whereas the organization of nanopits, or lack thereof in this specific case, was shown to both influence cell adhesion and osteogenic differentiation.[60] Surface curvature, particularly cell-sized concave features have been shown to facilitate cell spreading due to a promotion of increased intracellular tension. Cells will also preferentially migrate toward negative/concave regions, whereas positive/convex substrates present a diminished cell attachment and decreased migration speeds, proportional to decreasing feature diameter.[61,62]

Furthermore, the control over cell aspect ratio and subcellular curvature via geometrical restraints has shown interesting results in modulating not only cell shape but also lineage-specific differentiation. Similar to the effect of grooves where cell elongation is promoted, altering the aspect ratio from square to rectangular will render a similar outcome and stimulate osteogenic commitment. Subcellular curvature also plays a crucial role where adipogenesis is favored for large convex curves, whereas osteogenesis is promoted for concave edges and sharp vertices, which are linked to an increased intracellular tension. In fact, blocking select cell surface integrin receptors reduced intracellular tension and led to an inhibition of osteogenesis.[63] Studies on multicellular islands further highlighted the role of tension since cells located in convex edges preferentially committed toward the osteogenic lineage and cells on concave edges underwent adipogenesis. Interestingly, a similar trend was observed in a 3D hydrogel context where cell location on the high-tension construct periphery or low-tension center led to osteogenic or adipogenic differentiation, respectively.[64]

Taken together, these factors highlight the relevance of applying mechanical and physical cues to control cell behavior, that do not necessarily need the use of large quantity of biomaterials. Considering that cell sensing of the mechanical properties of its underlying substrate occurs at an approximate depth of 5 µm, depicting only a moderate response up to 20 µm, minimalistic scaffold approaches relying on thin structures or nano/micrometric topographical and geometrical cues are in fact attractive options.[65,66]

The above-mentioned aspects, on how biomaterials influence cell behavior, are highly dependent on surface and local mechanical topographical and geometrical properties. Such factors are in line with low material content platforms or devices, where the presence of such cues is imperative to guide cell migration, function, and organization. Contrarily, properties such as porosity, degradation, mechanical performance, namely viscoelasticity and stiffness, and the release of absorbed biomolecules are highly dependent on the bulk properties of the scaffold. As schematically represented in **Figure 2**, a higher number of surface properties, and thus with low dependence on the amount of scaffold material, can be pointed out comparatively to bulk properties. This reinforces the concept of "minimalist-engineering" approaches that have resulted in a multitude of low-material based bioengineered systems. The main idea is to fine-tune the spatial balance occupied by the inanimate region of the regenerative niche toward maximum actuation of the key living components during the healing process.

5. Low Material-Based TE Strategies Aiming Tissue Healing

Concerns related to "over-engineering" approaches and their effect on hampering the innate self-organization of cells have inspired biomaterials specialists toward the development of

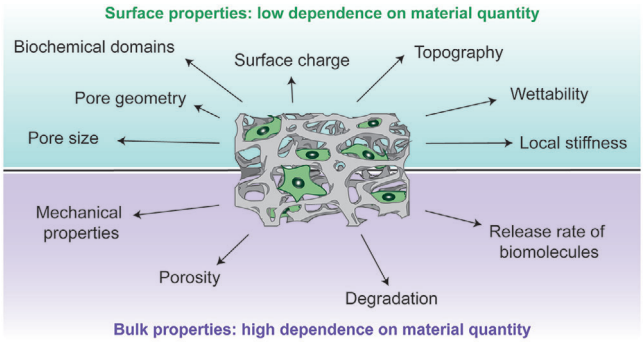


Figure 2. Surface versus bulk properties of biomaterials for tissue regeneration. Surface properties, such as pore size and geometry, exposure of biochemical domains to guide cellular behavior (e.g., signaling biomolecules, growth factors, drugs, and reprogramming factors), topography (e.g., random or aligned patterns, roughness), surface chemistry, including charge and wettability, and local stiffness, have low dependence on the quantity/volume of material used to produce the bioengineered scaffold. On the contrary, bulk properties, such as mechanical performance, porosity, degradation, and the release rate of absorbed biomolecules, are highly dependent on the quantity/volume of material.

low-material based bioengineering systems. Herein, we highlight examples of such systems, which, according to their complexity and architectural features, can be divided into a range of dimensions from "zero-dimensional" (0D) to "three-dimensional" (3D) approaches, as schematically represented in **Figure 3**.

5.1. Cell and Spheroid Engineering

Cells, either individual or in the form of spheroids, have been used as building blocks for the bottom-up construction of thick multilayered tissues resembling the complex and organized structure of native tissues. The rationale is to construct 3D structures with a precise control on the layer number, and cell type, distribution, and alignment. The 3D cell assembly by precise cell surface control was first reported by Bertozzi and co-workers[67] through complex biosynthesis and DNA conjugation. A simpler approach has been explored by Matsusaki and co-workers,[68] termed as cell accumulation technique (**Figure 4**A1). Using the layer-by-layer (LbL) technique, cells were coated with fibronectin and gelatin, and further assembled. After only 1 day, an ≈8 layered-construct was developed. The concept was validated with different cell types, namely human dermal fibroblasts, human umbilical vein endothelial cells (HUVECs), and human hepatocellular carcinoma cells (HepC2). Remarkably, for the case of HUVECs, fully and homogeneously vascularized tissues of 1 cm width and 50 µm height were obtained. This simple and rapid cell-accumulation technique was proposed as a promising *in vitro* technique for the construction of tissue or organ models. Comparing to other methodologies relying on the assembly of cells, such as classical cell sheet technologies, such cell-accumulation technique requires less time since the cell adhesion step is surpassed. Therefore, cell surface functionalization via LbL has proven to be a minimalistic-engineering approach which may overcome the limitations inherent to the use of bulk hydrogels, including limited cell–cell contact and signaling, as well as poor mass transportation of nutrients and metabolites.[69]

Similarly, spheroids have been also envisioned as building blocks for tissue engineering strategies. Each unit can mimic the physiological conditions during embryonic development, providing cell–cell/cell–matrix interactions within the 3D microenvironment, which is imperative to maintain intercellular functions.[70] One approach which significantly reduces the spheroidization time is the use of magnetic nanoparticles.[71] Through endocytosis, magnetic nanoparticles are internalized by cells, and then, the presence of a surrounding magnetic field allows the generation of spheroids. For instance, spheroids composed by MSCs and obtained by internalization of iron oxide nanoparticles exhibited absence of necrotic cores, while retaining the functionality of bone marrow niche.[72] Alternatively to cell internalization, the surface of the cell membrane can be functionalized with magnetic nanoparticles for the further spheroid fabrication. Recently, the magnetization of the cell membrane with poly(*L*-lysine), gold, and iron oxide nanoparticles was shown to produce islet spheroids with pancreatic β-cells and HUVECs.[73] The proposed strategy allowed the production of heterotypic pseudoislets with defined spatial distributions simply by applying a magnetic field through the spheroid. The best condition for an increased insulin secretion was achieved by β-cell composed pseudoislet surrounded by an outer layer of HUVECs, emphasizing again that cell–cell contact, and spatial distribution are essential for normal tissue function (Figure 4A2). Another strategy to reduce spheroid hypoxic core is to generate hybrid-spheroids with cells and artificial ECM fibrils. For instance, electrospinning yields fiber sheets, which can be degraded into individual microfibers via aminolysis. These structures pose as attractive vehicles for cell adhesion that can be modified with distinct moieties such as adenosine ligands, which have been singled out as key signals for induction of bone healing. Adenosine-presenting microfibers were specifically shown to mediate the formation of adi/stromal cell (ASC) aggregates, while directing osteogenic differentiation and inhibiting adipogenesis.[74]

Such tissue constructions using coated cells or spheroids were also explored as early versions of organoids to develop artificial organs for regenerative medicine or even organ replacement. The current challenge is mimicking the complex architectural macro features of native organs, and thus engineering organoids beyond the millimetric range, and in an organized assembly and acquisition of tissue function. Therefore, such strategies would greatly benefit with the combination of biomaterials technologies, as discussed in Subsection 5.5 Macrostructures.

5.2. Cell Sheets and Thin Films

Considering the state of the thin structures using low amounts of biomaterials, they can be basically divided into *i)* living or *ii)* inanimate structures. In the living structures subcategory, the thin morphology relies on the agglomeration of cells into a sheet-like conformance, known as cell-sheet technology. Cell sheets are scaffold-free strategies, thus relying on cell–cell junctions to form living engineered structures. The most well-characterized cell sheet technology relies on the use of thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm)-grafted surfaces, where cell monolayers can be conjointly recovered with its deposited ECM for tissue engineering strategies aiming toward ocular,

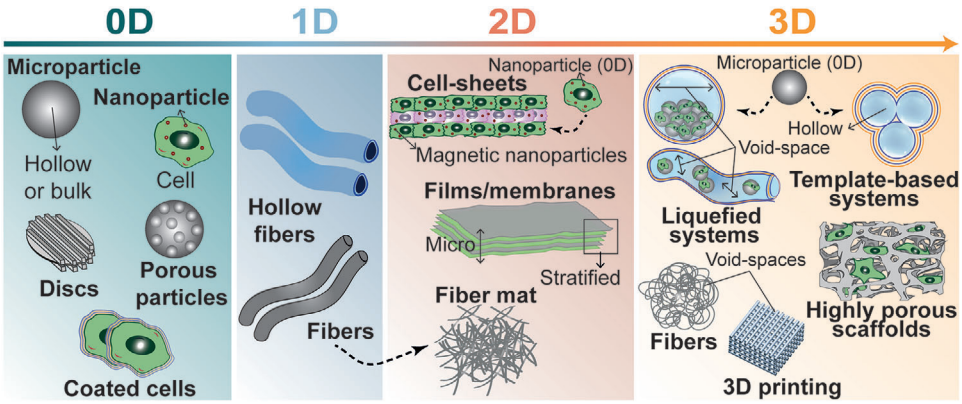


Figure 3. Approaches to engineer low-material based bioengineering systems according to their dimension ranging from "zero-dimensional" (0D) to "three-dimensional" (3D). Dotted lines connect each building block to the correspondent higher hierarchical order structure, namely i) microparticles (0D) can be incorporated within liquefied systems to provide cell adhesion cells or used to produce template-based systems (3D), ii) cells containing nanoparticles (0D) can be used to produce magnetic cell sheets (two-dimensional, 2D), and iii) fibers (1D) can be assembled to produce fiber mats (2D).

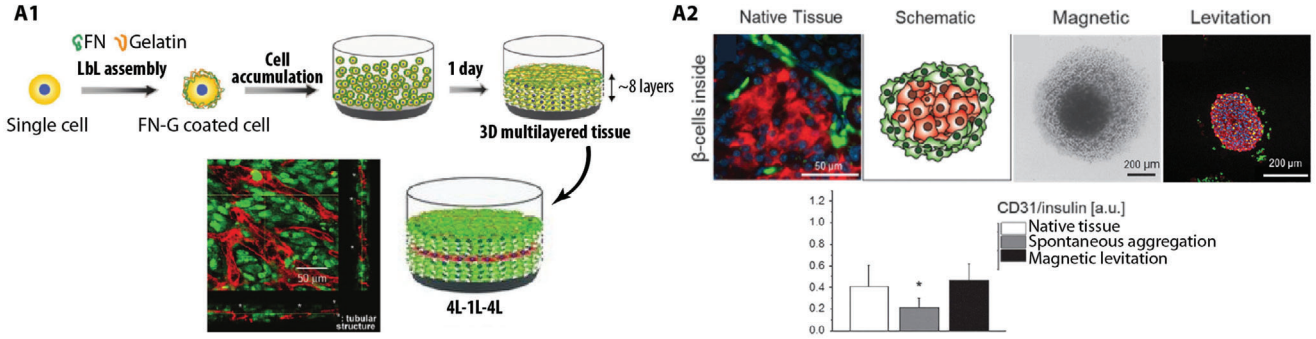
periodontal, bladder regeneration, among others.[75,76] Using the LbL technology, Matsusaki and co-workers prepared less than 10 nm thick fibronectin and gelatin films to fabricate multilayered tissue models, such as blood vessels, skeletal muscle, and connective tissue.[77] To produce multilayered tissues, such strategies rely on a previous cell adhesion step. In an attempt to reduce the time-consuming characteristic in the development of cell sheets, alternative techniques have been explored resorting for instance to magnetic properties. To this end, cell populations were labeled with magnetite cationic liposomes, rendering them susceptible to magnetic force and directing cell sheet formation. This strategy was applied to establish cocultures of fibroblasts with hepatocytes or ECs with hepatocytes, to form heterotypic cell sheets. Heterotypic cell sheets reportedly led to enhanced an albumin secretion when compared with homotypic sheets or heterotypic sheets with no applied magnetic force.[78,79] Relying on a similar approach, ECs and ASCs with internalized iron oxide nanoparticles were assembled in a stratified, hierarchical conformation where an EC sheet was formed between two ASC sheets (Figure 4B1).[80] This scaffold-free 3D strategy represented a step forward compared to two-dimensional (2D) traditional scaffold-free strategies, and did in fact aid in the recruitment of blood vessels and directed ASC differentiation into the osteogenic lineage. Moreover, cells can be assembled over not flat substrates permitting the construction of cell-sheet based complex structures.[81]

For the case of inanimate thin structures, multilayered systems have been proposed for a series of biomedical applications based on the LbL technology, that allows for the orderly assembly of nanosized layers with controlled final thickness and geometry.[82] Such strategies can find great application to compartmentalize cells, thus mimicking the native basement membranes which provides both structural and biological support to tissues. Aiming to mimic the basement membrane and its capability to compartmentalize cells, a nanometric collagen type IV and laminin multilayer was produced via LbL technology.[83] The synthetic basement membrane impeded cell migration while allowing for effective cell–cell crosstalk, as occurs in the native environment. Such inanimate thin membranes are a preeminent demonstration of the use of low quantity biomaterial to produce synthetic permselective biological barriers that could be useful to design new models for drug screening or engineering tissues for therapies.[84] With an opposing biological outcome, elastin, and collagen multilayers were also assembled by LbL to produce ECM-mimetic sheets.[85] Therefore, in contrast with the previous work, cells were expected to migrate and create a tissue featuring controllable ECM thickness and components. LbL is in fact an attractive option to envelop single cells and cell monolayers with thin multilayers.[86] Highly organized multilayers can be produced via LbL upon cell monolayers with natural ECM components, such as fibronectin and gelatin. The LbL nanometric membrane acts as an ECM mimetic adhesive surface, enabling cell monolayer build-up into a four-layered architecture for a fibroblast monoculture or coculture of endothelial and smooth muscle cells.[87] Alternatively, PLL-functionalized graphene oxide (GO) nanoparticles self-assembled into nanofilms have been applied as adhesive sheets for posterior cell deposition. This enabled the formation of interconnected multilayered cell constructs, featuring electrical conductivity and added mechanical properties. This system led to an improved cardiac cell organization, maturation, and cell–cell electrical coupling, with cardiomyocytes showing spontaneous beating and frequency-dependent opening/closing actuation under a low external electric field.[88]

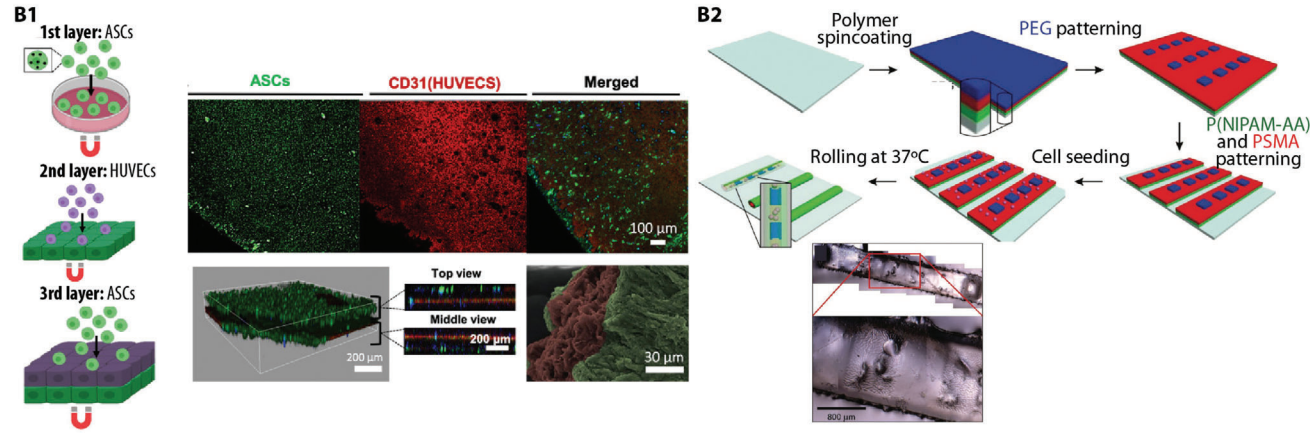
Thin multilayers can provide surface signals to guide cell behavior or they can even be used as reservoirs that could also play a therapeutic role in the regenerative process. For example, multilayers loaded with BMP-2 coating a simple hollow tube could trigger an effective new bone formation in a critical size femoral defect.[89] This clearly illustrates how devices with low material could have an impact in directing the regeneration of a "volumetric" tissue, such as bone.

Thin low-material based strategies can be further combined as building blocks to increase the architectural complexity of the system toward the development of stratified/multilayered macrotissues.[90] Alternatively, each thin building blocks can be functionalized and subsequently folded in an origami-based approach toward the development of multiform structures (Fig-

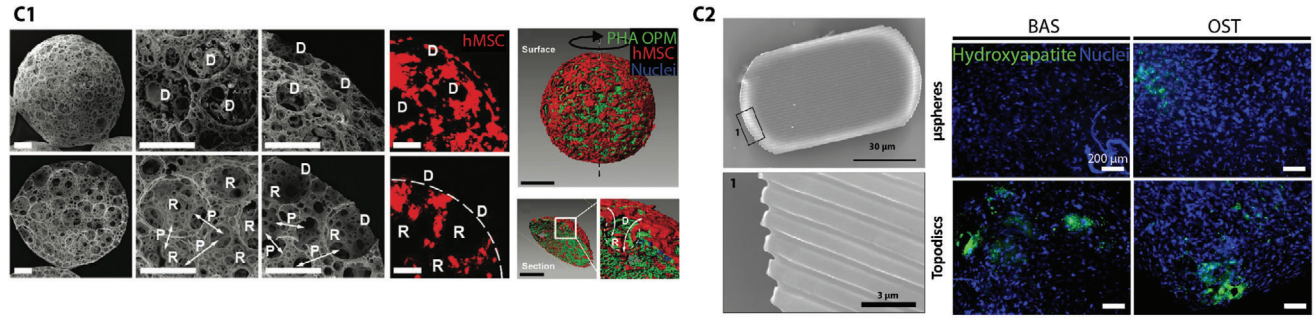
A. Cell and spheroid engineering



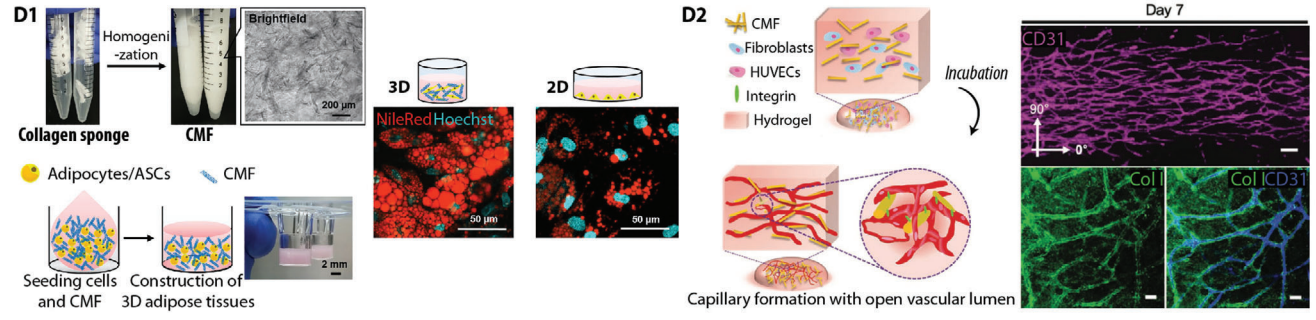
B. Cell sheets and thin films



C. Microparticles



D. Individual short-fibers



ure 4B2).[91,92] Either stratified or folded, when designing such structures the final thickness will dictate the timing for the establishment of a sufficient oxygen and nutrition supply. Therefore, it should be carefully considered since an absent or delayed vascularization is critical for the healing of a great number of tissue defects.

5.3. Microparticles

An increasingly explored strategy relies on the application of micrometric units, such as microparticles for a bottom-up 3D assembly while delivering cell function regulating cues. Microparticles can act as multifunctional tissue modulators due to the possibility to tailor both biochemical and, more importantly for the scope of this review, their biophysical properties. Whereas the option to control microparticle size is certainly an interesting feature, alternative biophysical aspects, such as surface area-to-volume ratio, geometry, topography, stiffness, and porosity take center stage in low-material strategies.[93] Given the emphasis of low-material strategies on minimizing nonessential bulk material, strategies to increase cell adhesion surface area gain relevance. Increasing microparticle porosity via, for instance, double emulsion assisted with gas successfully yielded highly open porous microspheres. Here, the combination of large pore surfaces and interconnected passages promoted hMSC proliferation and osteogenic differentiation, while also presenting promising results in an ectopic bone-formation mouse model by protecting cells from damage incurred during injection (Figure 4C1).[94] Another interesting approach relied on freezing an emulsion to prepare aligned-porous microspheres.[95] By unidirectionally freezing the emulsion, aligned solvent crystals were formed, which were then removed via freeze-drying to yield microspheres with aligned porosity. This strategy certainly presents itself as an interesting option to introduce geometrical cues and possibly to induce cell alignment within the microparticle structure, for example. Another alternative to direct cell alignment is via surface topographical cues such as grooves. To this end, nanogrooved microdiscs (Figure 4C2), named "topodiscs," were recently proposed, featuring an enhanced surface area to volume ratio comparatively to spherical microparticles and osteoinductive surface nanogrooves.[96] In fact, osteogenic differentiation of ASCs was promoted even in the absence of supplemental osteoinductive culture medium factors. Via a combination of stop-flow lithography with partial curing, nonspherical poly(ethylene glycol) diacrylate microparticles with wrinkled topography could be produced.[97] By partially curing the superficial polymer layer, wrinkling was induced by plasma treatment, which reportedly enhancing cell adhesion. There are, however, a sparse number of reported options to introduce topographical cues into microparticles for bottom-up engineering. Focusing on geometry, a comparison study between cuboidal and donut-shaped microparticles as cell microcarriers did not demonstrate a shape-dependent cell viability.[98] Whereas donut-shaped microparticles were expected to increase diffusion within the formed construct due to the introduction of a higher pore volume, experimental results did not corroborate this hypothesis.

Tailored microparticles do in fact present themselves as interesting cell carriers. First, as building blocks for bottom-up tissue engineering strategies, they enable an unconstricted cell organization and production of a functional histoarchitecture, as opposed to conventional bulk scaffolds.[99] Second, the myriad of production methods available imply that the selected microparticle may be fine-tuned for each individual objective. More importantly, these vast techniques allow us to take a step back from conventional spherical microparticles, with their immense unexploited volume, and take a step toward innovative microparticles with features, such as ultrathin thickness, augmented porosity, goal-oriented topography, and inventive geometrical cues.

5.4. Individual Short-Fibers

Produced via distinct techniques, such as electrospinning, wet spinning, interfacial complexation, and ranging a vast array of biomaterials,[100] the well-established nano/microthickness fibermat structures can be cut, either through mechanical processes, namely using microtome apparatus,[101] or chemical processes, such as alkaline hydrolysis[102] or aminolysis reaction,[74] giving raise to individual short-fibers. While mechanical processes using semi/automatic apparatus, such as microtomes can better control the size of the individual short-fibers, thus resulting in an enhanced size-distribution population, chemically processes present much higher yield rates, being thus more often explored.

Figure 4. Strategies for the development of minimalistic tissue engineering approaches aiming tissue healing, including i) cells and spheroid engineering, ii) cell sheets and thin films, iii) microparticles, and iv) individual short fibers. A) Cell and spheroid engineering – A1. Schematic representation of the bottom-up approach using layer-by-layer coated single cells to build 3D multilayered tissues. Confocal microscopic image shows the tubular endothelial structures (anti-CD31 antibody, red) developed after 1 week of incubation. Dermal fibroblast cells are counterstained with cell tracker green (green). Reproduced with permission.[68] Copyright 2011, Wiley-VCH GmbH. A2. Pancreatic β-cells (red) and HUVECs (green) distribution in native pancreas tissue, followed by representative brightfield and immunofluorescence images of heterotypic pseudo-islets formed with magnetic levitation. Magnetic levitation significantly increased HUVEC's integration ($*p < 0.01$, $**p < 0.001$). Reproduced with permission.[73] Copyright 2020, Mary Ann Liebert, Inc. B) Cell sheets and thin films – B1. Schematic illustration of production methods and confocal images of triple layer heterotypic cell sheet composed of a HUVEC cell sheet (red) between two ASC cell sheets (green). Reproduced with permission.[80] Copyright 2020, Elsevier B.V. B2. Scheme of production of tubular structures featuring 3D cellular patterns using shape-changing polymer films and corresponding brightfield image of F3T3 cells on an unfolded poly(N-isopropylacrylamide) (PNIPAM)-poly(styrene-co-maleic anhydride) (PSMA) film with polyethyleneglycol (PEG) pattern. Reproduced with permission.[92] Copyright 2018, Wiley-VCH GmbH. C) Microparticles – C1. Scanning electron microscopy (SEM) images of surfaces and sections of highly open porous microspheres (PHA OPM) and corresponding confocal images and 3D reconstruction of hMSCs adhered to PHA OPM. Reproduced with permission.[94] Copyright 2018, Wiley-VCH GmbH. C2. SEM image of nanogrooved microdiscs, "topodiscs", which induce tissue mineralization (hydroxyapatite in green) in the absence of osteoinductive factors. Reproduced with permission.[96] Copyright 2019, The Royal Society of Chemistry. D. Individual short-fibers – D1. Production of collagen microfibers and construction of 3D adipose tissues. 3D culture increased lipid storage versus 2D culture, as observed via Nile Red staining of lipid storage in adipocytes derived from ASCs after 21 days of differentiation, counterstained by Hoechst for nuclei. Reproduced with permission.[103] Copyright 2019, Elsevier. D2. Type I collagen microfibers applied for endothelial cell guidance and ultimate creation of open vascular lumen structure. Reproduced with permission.[104] Copyright 2020, Wiley-VCH GmbH.

Although substantially less explored compared to microparticles, such microfiber-long systems also pose as appealing tissue engineering supports for bottom-up approaches, that can be also modified with distinct moieties. Type I collagen microfibers (CMF-I) were recently proposed as optimal low-volume vehicles for ASC differentiation into the adipogenic lineage as well as in promoting phenotype maintenance of mature adipocytes, which is a challenging aspect due to rapid fat vesicle loss with conventional 2D culture (Figure 4D1).[103] This poses as an interesting result for cosmetic/pharmaceutical assays or even for plastic surgery substitutes. The potential of CMF-I to stimulate vascular tubular morphogenesis has also been assessed, where 20 µm long CMF-I were conjointly encapsulated with ECs within a fibrin hydrogel. Results showed a CMF-I-mediated cell alignment and the formation of a large vascular lumen, which did not occur in the absence of microfibers (Figure 4D2).[104] In another study, collagen type II microfibers (CMF-II) were used as spacers to controllably distance ATDC5 chondrogenic cells in an attempt to modulate chondrocyte differentiation via intercellular distancing. Type II collagen is a main component of the articular cartilage matrix and plays a role in inhibiting terminal differentiation of hypertrophy. Notably, increasing intracellular distance, which was also linked to an increasing elastic modulus, led to a decrease in cartilage-specific glycosaminoglycan, whereas the absence of CMF-II altogether led to an upregulated gene expression of cartilage markers.[105] Yet in contrast to microparticles, microfiber-long systems inherently classify as lower-material systems due to an enhanced area/volume ratio, while better mimicking the fibrous microenvironment of the native ECM found *in vivo*.

5.5. Macrostructures

5.5.1. Ultrathin Bulk Scaffolds

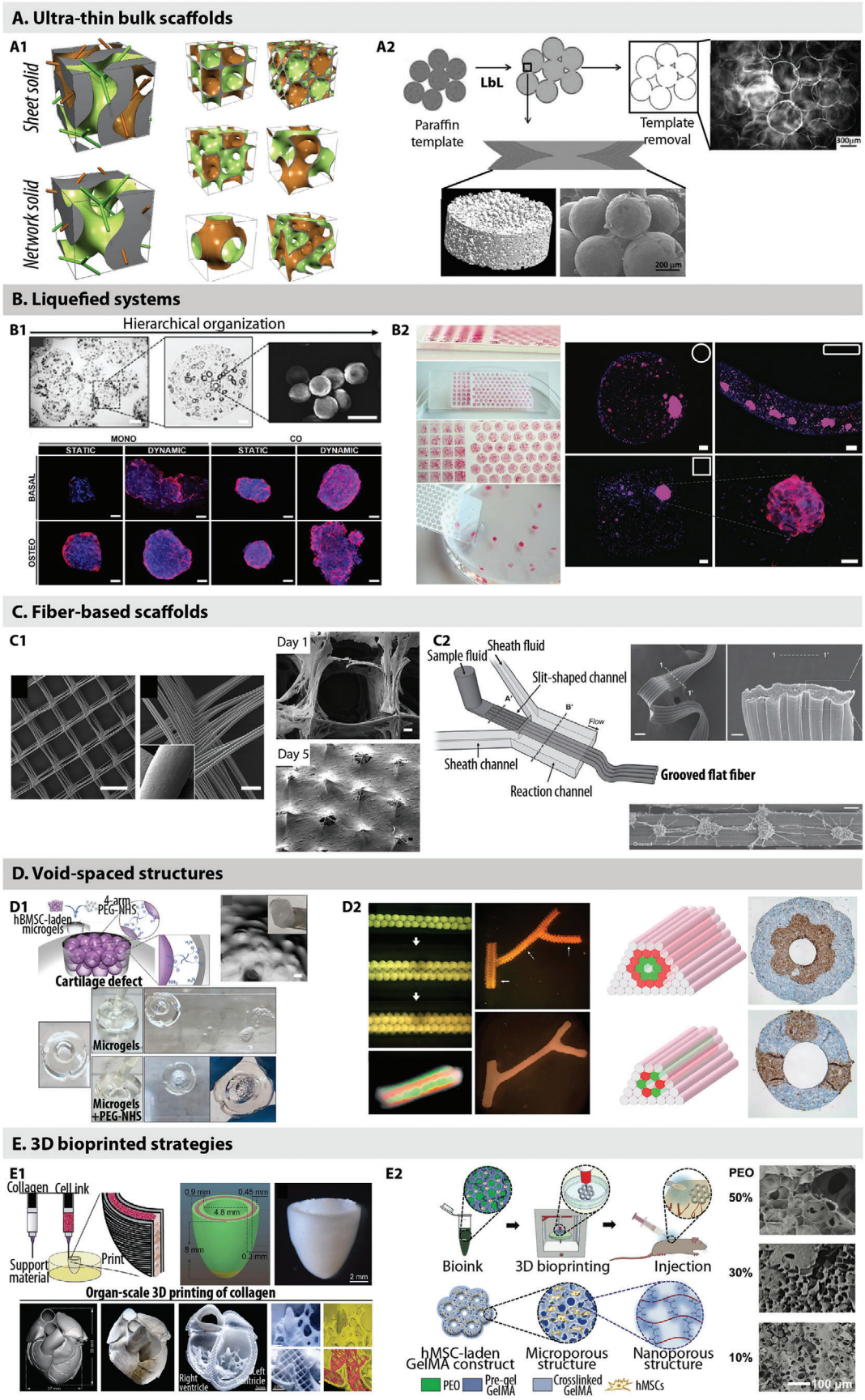
Bulk scaffolds are perhaps the most commonly applied structures for tissue engineering. Numerous variations have been proposed with combinations of distinct biomaterials, aiming toward the regeneration of alternative tissues. These top-down strategies however are hampered by a limited cell migration toward the scaffold's interior, may be subject to dimension-related diffusion issues, and commonly present a dense structure with a much higher biomaterial content than that actually required by the cells, in a sense that a high volume of the scaffold will not be populated with cells, thus leading to unfulfilled features of the engineered tissue.[98,106] Minimal material content scaffold design appears as an alternative design strategy to produce 3D structures bioinspired by biological tissues, such as beetle shells and crustacean skeletons. In these designs, a single solid domain is filled with an isotropic elastic material presenting either one interconnected void domain or two not interconnected void domains (**Figure 5**A1). While allowing for an increased cell migration, minimal material-content scaffolds are also characterized by their lightweight and high structural stiffness, rivalling that of conventional porous scaffolds.[107,108] In the minimal material-content scaffold design strategy, the increased porosity of scaffolds leads to an inherent reduction in wall thickness. Similar strategies to develop thin-walled scaffolds include the use of the layer-by-layer technique. Here, microparticles were used as random sacrificial templates for the 3D deposition of polyelectrolytes. Upon leaching of the paraffin sacrificial core, the resulting 3D structure retained its morphology, suggesting that pore size could be dependent on the initial templates and thus tunable (Figure 5A2).[109] Similarly, this methodology successfully enabled the production of nanometric scale structures with excellent interconnectivity using alginate and chondroitin sulfate as the binding material. Despite the low-material content, scaffolds could be handled and applied for the 3D culture of chondrocytes or hMSCs for cartilage TE.[110]

5.5.2. Liquefied Systems

Taking advantage of the mild and physical crosslinking of alginate, we have explored the production of multilayered and liquefied capsules containing microparticles for regenerative medicine purposes.[69,111–116] The introduction of microparticles in the core environment act as anchorage points for cells, ensuring cell survival and proliferation,[111,69] and, if desired, cell differentiation.[112] Such strategy relies on the use of alginate templates for the encapsulation of cells and microparticles within an ultrathin LbL membrane. Once the core is liquefied, cells can move freely and self-assemble into 3D aggregates. The void-spaces at the core of such capsules are also responsible for maximizing the exchange of important biomolecules for cell survival. Taking advantage of the liquid core, dynamic culture environments could be simply achieved using spinner flasks, thus without requiring complex bioreactor apparatus.[116] With that, we could stimulate the encapsulated ASCs to differentiate toward the osteogenic lineage in the absence of osteogenic differentiation factors. Additionally, combined with hydrodynamic atomization, an array of such liquefied-core capsules presenting different diameters in the micrometric range could be achieved at high rates (Figure 5B1).[114–116] We have also combined LbL with superhydrophobic–superhydrophilic microarrays or electrodeposition techniques to produce liquefied capsules with varying geometries (Figure 5B2).[117] Others, have developed compatible donut- and dumbbell-shaped liquefied microcapsules that enabled construct self-locking due to the LbL-induced shrinkage, followed by liquefaction-induced microcapsule swelling.[118] Such multigeometrical strategies may find great applicability in modular tissue engineering approaches. Alginate-based microparticles[119] and fibers[120] were also applied to produce analogous cell-laden, liquid core, freeform 3D macrostructures featuring alginate/chitosan multilayers.

5.5.3. Fiber-Based Scaffolds

Fiber-based scaffolds, which also meet the criteria of thin-walled structures with inherently increase porosity, have been produced via techniques, such as melt electrowriting (Figure 5C1) [121] and wet spinning.[122] Melt electrowriting in particular enabled the fabrication of highly ordered box-pore polycaprolactone (PCL) scaffolds that, upon coating with an ECM suspension derived from human decellularized adipose tissue, demonstrated an increased adipoinduction of hMSCs.[121] In turn, randomly oriented CHT/PCL based fiber mesh scaffolds successfully directed



neo-cartilage formation, dependent on the CHT/PCL ratio.[122] Microfluidic spinning permitted the production of flat alginate-based fibers featuring microgrooves, directing myoblast and neuron cell alignment (Figure 5C2).[123] Hollow microfibers have also been reported in the literature, produced via three-layered coaxial flow microfluidics, where the diameter of the alginate hollow fibers could be controlled by the flow rate. To demonstrate the potential of these structures toward the fabrication of a vascular construct, EC-laden hollow fibers were inserted and cocultured within a hydrogel structure containing smooth muscle cells.[124]

Given the fiber structure, the impact of external mechanical stimuli such as stretching, bending, and twisting may be easily assessed. Microfibers direct cell alignment due to their linear morphology and reduced diameter, which poses as an attractive feature to control cellular response. This effect can be further enhanced by the presence of grooves, with consequent activation of mechanotransduction pathways.[125] Taken together, microfibers may be efficiently tailored to meet desired requirements and direct the formation of a functional histoarchitecture.

5.5.4. Void-Spaced Structures

The production of hydrogel-based soft structures using techniques, such as laser ablation,[126] porogen introduction,[127] or template removal[128] to increase pore size or create channels has gained growing interest. Strategies to incorporate oxygen microbubbles include the straightforward foaming of methacrylated gelatin, which can then be UV crosslinked, and laden with cells.[129] Hydrogel bead-based scaffolds are another alternative for the development of porous macrostructures due to the intrinsic porosity formed between microbeads upon chemical bonding. An example is the application of a 4-arm polyethyleneglycol (PEG)-*N*-hydroxysuccinimide crosslinker for a spontaneous adhesion of cell-laden, norbornene-functionalized gelatin microbeads into a robust, porous structure (Figure 5D1).[130] Bead-based scaffolds can also be combined with the porogen removal technique to further tailor scaffold complexity. To this end, PEG-based structural and noncytotoxic porogenic microspheres were assembled conjointly with cells to form a macrostructure with controlled porosity upon porogen removal, presenting a homogenous cell distribution throughout the resulting structure. Scaffold formation occurred via interaction of amines present in serum proteins originating from the cell culture medium with vinylsulfone groups present on the microsphere's surface. Furthermore, the density of porogenic microspheres could be tuned to form scaffolds with gradients of macroporosity. Further functionality could be introduced by delivering angiogenesis-promoting molecule sphingosine 1-phosphate (S1P) within a third set of PEG/BSA microspheres.[127]

Template removal strategies have been applied to improve diffusion within the hydrogel's macrostructure or as alternatives for vasculature engineering. An option is the creation of a needle-guided path within a hydrogel microfluidic set-up, followed by endothelial cell seeding to recapitulate a biomimetic human endothelial lumen.[131] A more complex structure envisioned the 3D printing of an open, interconnected carbohydrate-glass lattice, which was subsequently enveloped by a cell-containing fibrin hydrogel. Upon mild lattice dissolution with media perfusion, the resulting 3D network could be lined with endothelial cells and perfused with blood.[128] Aiming toward a similar outcome, sequential 3D printing of collagen layers interweaved with sacrificial gelatin channels was performed, where gelatin was posteriorly removed and the formed channels could be perfused with media.[132] The development of a scaffold-free vascular structure envisioned the 3D printing of multicellular cylinders, which were supported by temporary agarose rods until multicellular cylinder fusion, after which rods were removed and the final construct collected. The technique enabled the formation of a double-layered vascular wall featuring a coculture of HUVECs and fibroblasts, which could be assembled in a linear or branched architecture (Figure 5D2).[133]

5.5.5. 3D Bioprinted Strategies

The 3D bioprinting technology brought key improvements to organoid-based strategies by providing a better control over tissue size and architecture to ultimately develop artificial organs for regenerative medicine or even organ replacement. Recently, a strategy was proposed based on stem cell and organoid fusion and reorganization, restricted by 3D printed geometries and

Figure 5. Macrostructured strategies for the development of minimalistic tissue engineering approaches aiming tissue healing. A) Ultrathin bulk scaffolds — A1. Network versus sheet solid scaffold architectures and examples of triply-periodic minimal surfaces. Reproduced with permission.[107] Copyright 2011, Elsevier B. V. A2) Representation of the production method for liquefied 3D constructs with corresponding microtomography and SEM images of multilayered core material and fluorescence images of 3D structure upon core leaching. Reproduced with permission.[109] Copyright 2010, Wiley-VCH GmbH. B) Liquefied systems — B1. Hierarchical organization of multilayered liquefied-core microcapsules and their application as vehicles for the osteogenic differentiation of adipose-derived mesenchymal stem/stromal cells (ASCs) (osteopontin in pink counterstained with nuclei in blue). Reproduced with permission.[116] Copyright 2019, IOP Publishing. B2. Attached and detached square- and circle-shaped liquefied capsules containing microparticles stained with Nile red. ASCs cultured within liquefied capsules at 7 days (F-actin in pink, nuclei in blue). Reproduced with permission.[117] Copyright 2020, Wiley-VCH GmbH. C) Fiber-based scaffolds – C1. SEM images of highly ordered box-pore scaffolds alone and with seeded hMSCs on days 1 and 5. Reproduced with permission.[138] Copyright 2020, Wiley-VCH. C2. Schematic representation of production method for flat microgrooved microfibers. SEM images of flat microgrooved microfibers, corresponding cross-section, and neuron seeded microfiber. Reproduced with permission.[123] Copyright 2012, Wiley-VCH GmbH. D) Void-spaced scaffolds – D1. Microgel assembly and microgel-tissue integration, enabled using the microgel/PEG-NHS pairing. Reproduced with permission.[130] Copyright 2018, Elsevier. D2. Spheroid-based fabrication of tubular structures based on cell fusion using removable tubular structures. Double-layered vascular wall containing human umbilical vein smooth muscle cells (green) and human skin fibroblasts (red) multicellular cylinders with smooth muscle α-actin stained in brown. Reproduced with permission.[133] Copyright 2009, Elsevier. E). 3D bioprinted strategies – E1. Schematic representation of dual-material printing of a ventricle model using collagen (green and yellow) and high-concentration cell-based (cardiac cells in pink) inks with a central section of cardiac cells (pink) and printing of human neonatal-sized heart. Reproduced with permission.[139] Copyright 2019, AAAS. E2. Schematic representation of production method and application of 3D-bioprinted hierarchically porous hydrogel constructs using an aqueous two-phase bioink, with corresponding alteration of porous structure by varying PEO volume fractions. Reproduced with permission.[137] Copyright 2020, Wiley-VCH GmbH.

constraints.[22] By controlling the geometry and cellular density, centimeter-scale tissues enabled the self-organization of lumens, branched vasculature, and tubular intestinal epithelia with crypts and villus domain within a Matrigel hydrogel. Given the fact that low-material based systems are often gravity defying structures, viscoelastic supporting baths have been also proposed.[134] Additionally, in order to increase the resolution of 3D bioprinted structures without limiting the materials that can be printed, freeform supporting baths have also gained considerable interest. A remarkable example is the 3D-printed collagen structures resembling structures of the human heart using reversible embedding suspended micrometric hydrogels (Figure 5E1).[135] In order to bypass the requirement of a biomaterial-based supporting bath, another technique was developed, which relied on iPSC-derived organoids as supporting elements. Via sacrificial writing into the functional organoid-based tissue and subsequent removal, perfusable channels were formed throughout the structure to yield bulk vascularized tissues. Promising results were obtained with the production of a synchronously beating cardiac tissue up to 7 days, underlining the potential of this technique to be broadened for the fabrication of distinct tissues.[136] We foresee more advances toward hierarchical low-material based strategies, where for example the bioink porosity can be combined with porosity given by the geometry of the printed constructs (Figure 5E2).[137]

Over the past few decades, macrostructures for tissue engineering have received a substantial makeover. Initially dependent on top-down fabrication techniques with inherent limitations related to homogeneous cell seeding, diffusion, and the viability to mimic a functional histoarchitecture, recent advances have opened up a multitude of possibilities and strategies to overcome these limitations. Furthermore, these advances often come hand in hand with a reduction in material content, as low material-based strategies. By designing minimal material-content scaffolds specific properties can be easily enhanced maximizing the bioperformance of such scaffolds. For example, increasing the density of porogens the porosity of the scaffold can be easily increased, thus increasing the diffusion of essential molecules for cell survival. Additionally, augmented void-spaced macrostructures can also provide more cell adhesion sites due to enhancement of surface area, while also reducing the quantity of unnecessary material, which could ultimately constrict cell movement. Fiber- and bead-based strategies as well as liquid-core structures/scaffolds present themselves as options to perform this, yet organization of porosity is generally random. Template removal strategies and approaches to include perfusable channels within macrostructures have thus been increasingly explored in order to bypass diffusion issues attributed to scaffold size, where channel location can be actively controlled. A combination of soft and stiff substrates while still striving toward optimal nutrient/waste diffusion may also be applied. A strategy for facile intraperitoneal implantation and retrieval aiming toward type I diabetes cell replacement therapy involved the development of interconnected toroidal-shaped structures. The flexible 3D-printed double-helix resin structure acted as a reinforcement for the rat islet equivalent-containing alginate hydrogel, conferring mechanical support while guaranteeing the required mass transport conditions attributed to the high surface area to volume ratio. Diabetes correction was observed up to 12 weeks in a diabetic mouse model, providing a proof-of-concept for the application of this intricate cell-based technique.[138] It would thus be interesting to explore alternative, biodegradable materials for the production of the flexible reinforcement structure, while retaining the attractive mechanical properties that allow for device retrieval.

Figures 4 and 5 summarize the above-referred examples of multiple low material-based TE strategies aiming towards tissue healing.

The above-referred examples of low-material based TE strategies that aim to establish an appropriate biomaterial-cell content balance are summarized in **Table 1**. The preclinical/clinical application of such strategies are also highlighted.

6. Conclusions and outlook

In the past decade, we have been witnessing a paradigm shift and a tremendous progress in the TERM field, including the discovery and characterization of new stromal/stem cell sources, and the development of smart and dynamic biomaterials with tunable properties. Several major challenges have been thus addressed, namely the mitigation of host responses through immunomodulation, and improved vascularization strategies. In fact, one of the major problems hampering the clinical outcome of TE strategies is the absence of a functional vascular supply or its delayed establishment.[141] As a consequence of being deprived from the exchange of essential molecules, including the inflow of nutrients and oxygen, as well as the release of waste metabolites, cell death occurs. Additionally, cell–cell interactions may be blocked, hampering the exchange of important cell signaling molecules indispensable in the orchestration of cellular crosstalk events toward tissue healing.[142] Such blockages can be resultant from the presence of a physical barrier imposed by an excessive in situ volume of engineered material implanted into the defect site, and/or due to an excessive amount of ECM, when considering strategies that rely on a preseeding approach. Nevertheless, physical barriers block the ingrowth of host vessels and thus prevent the establishment of vascular supply. These physical constraints surpass the proangiogenic strategies often imprinted into the biomaterial, thus explaining the major discrepancy between scientific research efforts and the clinical applications of such strategies. Concerns related to "over-engineering" approaches and their effect in hampering the innate self-organization that all organisms, including humans, are capable of, have also been recently pointed out by others.[7,143] Our main message herein assets in a "minimalist-engineering" approach relying on minimizing of the amount of implanted biomaterials, as opposed to an excessive in situ overloading that ultimately leads to the failure of the TE strategy. Of note, we are aware of the great challenge in defining such minimal amount, and thus low-material based approaches should be carefully analyzed and take into consideration tissue- and biomaterial-specific factors, ranging from the physicochemical characteristics of the damaged tissue, mechanical loading, resident cell characteristics, namely proliferation ratio and density, biodegradation, as well as the extent of the lesion. Upon implantation, the "minimalist material engineering" approaches herein proposed will be greatly beneficial if designed to work in tandem with recruited cells, including endogenous progenitor or stromal/stem and immune cells. For that, low-material based bioengineered systems can express surface

Table 1. Examples of low-material based strategies and their preclinical/clinical application.

Low material-based strategies	Subtype	Methodology	Preclinical/clinical application	Ref.
Cell and spheroid engineering	Single cells	Layer-by-layer of single cells (HUVECs and HepC2) with fibronectin and gelatin	Development of layered vascularized tissues (1 cm width and 50 μm height).	[68]
	Spheroids	Cell magnetization via iron oxide nanoparticle internalization	MSC spheroids.	[72]
		Cell membrane magnetization with PLL, gold and iron oxide nanoparticles	Increased insulin secretion from islet spheroids composed of pancreatic β-cells with an outer HUVEC layer.	[73]
		Incorporation of polydopamine-assisted adenosine coated microfibers	Enhanced *in vivo* bone regeneration in calvarial defect model.	[74]
Cell sheets and thin films	Cell sheets	Application of thermoresponsive PIPAAm-grafted surfaces	Transplantion of corneal epithelial cell sheet for human corneal regeneration. Periodontal ligament cell sheet transplantation in athymic rat periodontitis model. Urothelial cell sheets to replace gastrointestinal mucosa in gastric flap for application in a surgical dog model of bladder reconstruction.	[75,76]
		Layer by layer of SMC and HUVEC cell sheets with fibronectin and gelatin	Production 3D blood vessel constructs similar to native rat artery.	[140]
		Cell labeling with magnetite cationic liposomes	Enhanced albumin secretion by HepG2 cells within heterotypic mixed NIH3T3 and HepG2 cell sheets under magnetic force.	[78,79]
		Internalization of iron oxide nanoparticles by ECs and ASCs for magnetic-force dependent production of stratified cell sheets	Increased blood vessel recruitment and osteogenic differentitation of ASCs.	[80]
	Thin films	LbL with collagen type IV and laminin	Basement membrane-mimetic structure to impede cell migration while enabling cell-cell crosstalk.	[89]
		LbL with elastin and collagen, or fibronectin and gelatin	ECM-mimetic cell sheets for 3D microtissue construction.	[85,87]
		Self-assembly of PLL-functionalized graphene oxide (GO) nanoparticles into nanofilms	Electrically conductive nanofilms for promotion of cardiac cell organization, maturation, and cell-cell electrical coupling within multi-layered constructs.	[88]
		BMP-2 loading of PLL and hyaluronic acid LbL film on PLGA hollow tube	Increased bone regeneration in a critical size femoral defect.	[89]
Microparticles	Highly open porous polyhydroxyalkanoate microspheres	Double emulsion assisted with gas	Increased bone formation in ectopic bone-formation mouse model.	[94]
	Aligned-porous PCL microspheres	Unidirectionally freezing of emulsion followed by freezedrying	Cell microcarriers with added possibility of cell alignment within the microparticle structure.	[95]
	Nanogrooved microdiscs	Micromoulding of PCL microspheres	Topography-prompted osteogenic differentiation of ASCs.	[96]
	Wrinkled surface microparticles	Stop-flow lithography with partial curing using PEGDA	Enhanced cell adhesion.	[97]
	Cuboidal and donut-shaped microparticles	Poly(d,l-lactic acid) imprinting	Cell microcarriers.	[98]
Individual short-fibers	Microfibers	Collagen type I sponge homogenization	ASC differentiation into the adipogenic lineage and promotion of phenotype maintenance in mature adipocytes.	[103]

(*Continued*)

Table 1. Continued.

Low material-based strategies	Subtype	Methodology	Preclinical/clinical application	Ref.
		Collagen type II sponge homogenization	Incorporated within fibrin hydrogels with ECs to direct for formation of a vascular lumen. Applied as spacers to controllably distance chondrogenic cells for intercellular distancing-based modulation chondrocyte differentiation.	[104,105]
Macrostructures	Ultra-thin bulk scaffolds	Build-up of LbL membrane composed of chitosan/chondroitin sulphate or PEI/chitosan/alginate surrounding packed paraffin microspheres as a sacrificial template	Applied for chondrogenic differentiation of hMSCs and chondrogenic maintenance of chondrocytes.	[109,110]
	Liquefied systems	Spherical, triangular, and quadrangular microcapsules composed of a liquefied alginate core enveloped by a PLL/alginate/chitosan LbL membrane	Dynamic cell culture environment due to liquefied core containing microparticles as cell anchorage points.	[114–116] [117]
		Donut and dumbbell-shaped microcapsules	Microcapsule self-locking capabilities.	[118]
		Reeled liquefied-core alginate fiber enveloped by alginate/chitosan LbL membrane	Sustained cell expansion vehicle.	[120]
	Fiber-based scaffolds	Box-pore PCL scaffolds produced via melt electrowriting	Increased hMSC adipoinduction.	[121]
		Randomly oriented CHT/PCL-based fiber mesh scaffolds	Neo-cartilage induction dependent on CHT/PCL ratio.	[122]
		Flat, microgrooved alginate-based fibers	Induced alignment of myoblasts and neuron cells.	[123]
		Hollow alginate microfibers produced via three-layered coaxial flow microfluidics	Strategy for production of vascularized tissues via embedding of endothelial cell seeded hollow fibers into smooth muscle cell-laden agar-gelatin-fibronectin hydrogels.	[124]
	Void-spaced structures	Foaming of cell-laden methacrylated gelatin followed by UV crosslinking	Sustained ASC viability and phenotype maintenance within large-sized constructs.	[129]
		Surface chemistry mediated crosslinking of cell-laden gelatin microbeads	Enhanced chondrogenic differentiation of hMSCs within microgel-based scaffolds vs bulk scaffolds.	[130]
		Porogen removal for development of PEG scaffold with macroporosity gradients	Delivery of angiogenesis-promoting molecule sphingosine 1-phosphate.	[127]
		Retractable needle-guided path within hydrogel	Recapitulate a biomimetic human endothelial lumen.	[131]
		3D printing of an open, interconnected, sacrificial carbohydrate-glass lattice, enveloped by a cell-containing fibrin hydrogel	Perfusable endothelialized 3D structure.	[128]
		3D printing of collagen layers interwoven with sacrificial gelatin channels	Perfusable 3D structure.	[132]
		3D printing of multicellular cylinders combined with retractable agarose rods	Production of hollow double-layered vascular wall featuring a co-culture of HUVECs and fibroblasts.	[133]
	3D bioprinted strategies	3D printing of organoid-forming stem cells in constrained geometries and at controlled cellular densities for spontaneous self-organization	Self-organization mediated production of intestinal tubes.	[22]
		3D printing using a freeform supporting bath composed of micrometric hydrogels	Fabrication of collagen constructs resembling structures of the human heart.	[135]
		3D printing using a freeform supporting bath composed of iPSC-derived organoids	Sacrificial writing of perfusable channels within supporting iPSC-derived organoids for production of a synchronously beating cardiac tissue.	[136]

biochemical cues to facilitate or suppress cellular reprogramming toward a specific differentiation route, and thus, matching the type of tissue aiming to be regenerated. For example, epigenetic drugs have been successfully applied for scaffold manipulation, such as in muscle[144] and tendon TE,[145] or in the development of powerful *in vitro* platforms for disease modeling, such as cardiac disease,[146] periodontis,[147] and osteoarthritis.[148] Likewise, immunomodulatory cues may aid to orchestrate the kinetics of the recruited immune cells toward tissue remodeling and healing. Therefore, the highly dynamic immune environment surrounding such immunomodulatory biomaterials would favor a more proregenerative immune response. Consequently, the bioperformance of the implanted biomaterial would be favored, thus resulting in a highly efficient regenerative process. For that, *in vitro* platforms able to predict the immunomodulatory bioperformance of biomedical devices following implantation are of the utmost importance.[149]

Despite the great amount of knowledge and progress achieved during the past decades, the clinical translation of TERM strategies still faces a myriad of challenges, including regulatory and economic hurdles, intrinsically correlated with the size of the potential patient market. We believe that the concept herein debated of a "minimalist-engineering" approach, in particular through the use of low material-based bioengineered systems, circumvent the major translational drawbacks found in classical TE strategies. In particular, the use of the systems highlighted, namely cell-only spheroids, microparticles, and individual short-fibers, enable a bottom-up assembly upon *in vivo* implantation. Therefore tissue-engineered constructs with clinically relevant size can be rapidly achieved following implantation into the defect. Such modular processes are highly advantageous due to their scalability. Additionally, they can be implanted via minimal invasive procedures in a ready-to-use fashion or requiring low manipulation by the clinicians. For the remaining low material-based system highlighted, namely macrostructures, the translational challenges found for classical TE applications are unfortunately similar, with analogous regulatory affairs related to the type of biomaterial employed. However, the key difference lies in the amount of material used, which might consequently translates into more economically viable options and positively impacts scalability.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

low-material based strategies, self-organization, tissue healing

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