

Tissue Regeneration: From Synthetic Scaffolds to Self-Organizing Morphogenesis

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Abstract: Regenerative medicine offers therapeutic approaches to treating non-regenerative diseases such as spinal cord injury and heart disease. Owing to the limited donor tissue available, cell-based therapy using cultured cells with supporting scaffolds has been proposed to rebuild damaged tissue. Early attempts at repairing skin and cartilage achieved significant success thanks to the simplicity of the tissue architecture, which later fueled enthusiasm for applying the same strategy to other types of tissue. However, more complex tissue functions require a more extensive vasculature and heterogeneous cell arrangements, which together constitute a significant hurdle in practical applications. Accordingly, recent years an increased interest has been in the use of decellularized matrices that retain the natural microarchitecture as the scaffold. However, although a number of engineering approaches have been suggested, self-organizing behavior such as cell proliferation, migration, and differentiation may still disorganize and frustrate the artificial attempts. This mini-review first provides examples of the early history of tissue engineering using skin and cartilage as examples, and then elaborates on the key technologies used to fabricate synthetic acellular scaffolds and cell/scaffold constructs with more complicated architectures. It also summarizes the progress achieved in the use of decellularized matrices for cell seeding as well as the recent success seen in self-organizing two- and three-dimensional tissue formation with the aid of biomathematical modeling. The review concludes by proposing the future integration of biomathematics, developmental biology, and engineering in concert with the self-organization approach to tissue regeneration.

Keywords: Morphogenesis, pattern formation, regenerative medicine, scaffold, self-organization, tissue engineering.

INTRODUCTION

Tissue regeneration is a new and burgeoning field whose aim is to improve health and quality of life by restoring or repairing impaired tissue [1]. Demand for such regeneration is driven by the limited self-healing ability of most adult tissue following severe injury or chronic degeneration. For example, the lack of regenerative capability exhibited by cardiac muscle and nervous system is often associated with conditions such as heart failure, spinal cord injury, and Alzheimer's disease. Given this lack of self-healing ability, external assistance is required to facilitate the healing process or even to rebuild a replacement for damaged tissue. Clinical demand for such assistance has motivated research into tissue regeneration. In addition to clinical applications, the regenerated tissue construct can also serve as an *in vitro* model for testing drug efficacy and toxicity based on its morphological or functional analogy with native tissue. In other words, successful tissue reconstruction can provide an *ex vivo* model that resembles physiological behavior *in vivo*. Accordingly, it offers a potentially superior alternative to animal models, which suffer a number of disadvantages, including high costs, ethical concerns surrounding the sacrifice of animals, and failure to accurately predict human

responses owing to differences in species and physical scale [2, 3].

In recent years, there has been a surge in stem cell research to meet growing demand for cell sources to restore tissue function. Some of the results have shown promise for their capability of differentiation into many cell types. As such, many studies focus on defining the inductive factors that can lead stem cells to differentiate into specialized cell types. However, regeneration of functional tissue cannot rely on chemically induced differentiation alone. The desired properties for regenerated tissue may be structural, such as bone and cartilage, protective, such as skin and capillaries, or metabolic, such as liver and pancreas, which requires that constituent cells, soluble chemical factors, physical factors, and the structural template operate in spatiotemporal coordination with one another. For example, the alignment of cardiac fibers within multiple cardiac muscle layers is reported to be essential for heart contraction [4]. Similarly, a radial network of capillaries for the fluidic transport of metabolites has also been shown to play an important role in the biochemical and detoxification functions of hepatic lobules [5]. Positioning these cells appropriately, inducing them to deposit extracellular matrices, and organizing the elements spatially across several levels of larger tissue and organ structures requires the correct cues. Hence, considerable challenge remains to ensure orchestration among cells, the tissue structure, chemical factors (e.g., growth factor), and physical factors (e.g., cyclic mechanical force), when trying

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to implement tissue microarchitecture and recapture the complex organization.

It is well understood that tissue engineering, or regenerative medicine, requires multidisciplinary contributions to find the ultimate solution. Early tissue engineering was driven primarily by frustration over the limited supply of tissue available for clinical transplantation. This lack of functional tissue replacements resulted in the deaths of thousands of patients every year. Around the same period of time, new biomaterials entered the medical arena, subsequently resulting in the initiation of tissue engineering. However, the urgent needs for clinical applications rendered early studies largely empirical. In other words, rather than developing a strategic plan based on a fundamental understanding, early studies tended to rely heavily on existing materials and to strive for “know-how.” In the words of Ingber *et al.*, “early studies were largely empirical in design: put together your best ideas and materials, throw them in an animal, and pray for the best” [6]. Although these early trials achieved some success, most of them failed, and little understanding of tissue development was obtained.

With the advent of tissue engineering, however, developmental biology underwent a shift in focus from the physical process of tissue formation to the power of genetics. That is, developmental biologists beginning to search for the critical genes responsible for switching organ-specific morphogenetic programs, including the related signaling pathway, growth factor, and signal transduction cascade necessary for organ formation. However, most of the findings were restricted to the upstream. When considering an engineering strategy for the design of a tissue construct, the lack of sufficient links between the upstream gene and downstream developmental dynamics constitutes significant impediment to implementation of the biomimetic environment. This chasm has recently been bridged by developments in microtechnology and three-dimensional (3D) cultures, which have inspired approaches to investigating the governing factors in the physiological behavior of cells [7]. With the aid of micromechanical tools, researchers have shown that combined mechanical and biochemical activities and the interaction between cells and the extracellular matrix (ECM) are important to tissue development, such as changes in cell proliferation, migration, differentiation, and metabolism in response to force transmission or chemical gradients. Although such findings contributed many new insights, they also raised new questions. As cells interact with the extracellular environment during many activities, e.g., cell migration [8] and cell-cell alignment [9], attempts that use artificial controls in tissue reproduction, such as depositing cells in a specific location, may eventually become disorganized and frustrated by cellular organizing events. Engineers are facing challenges including not only the complications that arise in implementing tissue architecture, but also the self-organizing behavior of cells in developments subsequent to cell settlement.

Accordingly, tissue regeneration requires knowledge of the developmental process, the engineering technology needed to implement the regulatory mechanism, and, most importantly, synergic approaches that incorporate self-organization and enable a strategy capable of directing tissue formation toward the desired outcomes. This mini-review

provides an overview of engineering approaches to the control of tissue formation. The content of this review is organized as follows. The first section discusses the early history of tissue engineering using skin and cartilage as examples. It is followed by a broad overview of the state-of-the-art technologies for the geometric control of tissue, with emphasis on the manufacture of synthetic acellular scaffolds, the cell/scaffold construct, and the most recent progress achieved in the use of decellularized matrices. The importance of self-organizing morphogenesis to spontaneous tissue growth is then discussed in line with the success of biomathematics and 3D cultures of pluripotent stem cells. Fig. (1) presents a schematic illustration of the technologies available to guide tissue formation. The review concludes with recommendations based on existing approaches, suggesting the seamless integration of biomathematics, developmental biology, and engineering working in concert with tissue morphogenesis. It is hoped that the review will inspire new perspectives on tissue regeneration in future research.

EARLY TISSUE ENGINEERING: SKIN AND CARTILAGE

A brief history of tissue engineering can be found in Berthiaume *et al.* [10]. As previously noted, early developments in tissue engineering were driven by clinical demands. Skin and cartilage grafts are among the most representative tissue engineering treatments for acute injury and chronic disease. Skin grafts are the first engineered tissue construct, and were developed by Green *et al.* [11-13] and Bell *et al.* [14]. They are used for wounds that are more than 1 cm in diameter or that extend deep into the dermis, thereby requiring special treatment to assist in closure. Autologous skin grafts remain the gold standard [15], but the available donor sites for skin graft material are limited. Researchers thus optimized a protocol whereby cultured keratinocytes isolated from patients [11-13] were regrown *ex vivo* and expanded the coverage area a thousand-fold in a matter of weeks. This protocol was subsequently developed into a product called EPICEL, which is used to treat patients suffering from catastrophic burn injuries. In addition, researchers also found that keratinocytes can be cultured in artificially produced neodermis via allogeneic dermal fibroblasts in gel [14], resulting in another successful product, Apligraf®. However, because living cells are used, immunological rejection may occur in the case of allogeneic transplants.

An alternative type of skin graft uses a porous matrix placed at the wound surface, thus allowing the revascularization and migration of host cells such as fibroblasts, endothelial cells, and neural cells [16]. The ultimate goal of a graft is full integration with the host tissue. Thus, rather than providing a tissue replacement, this acellular skin graft approach is more akin to a structural template that stimulates the patient's repair and regenerative processes. It has also been proposed that decellularized donor skin can maintain structural stability while preventing an allogeneic immunological response [17].

Similar to skin grafts, the development of cartilage grafts also arose from clinical demands. A loss of cartilage function is often a result of degenerative joint diseases in the older population and sports injuries in the younger population. Cartilage has limited regenerative ability because it lacks the

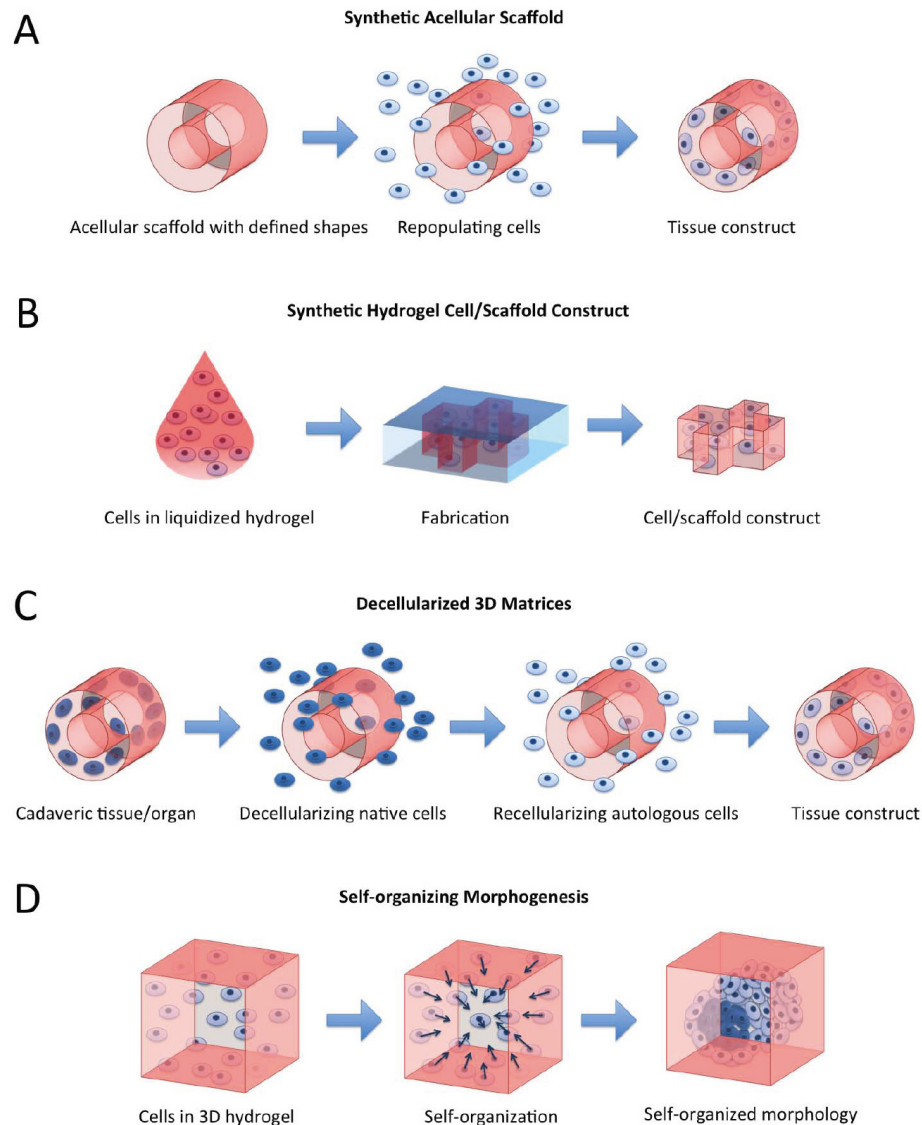


Fig. (1). Schematics of technologies available to guide tissue formation. **(A)** Synthetic acellular scaffold that uses a machining process to fabricate an acellular scaffold of a specific shape, followed by cell repopulation. **(B)** Synthetic hydrogel construct that works in liquidized hydrogel to fabricate the geometry of a hydrogel scaffold with cells encapsulated inside. **(C)** Decellularized 3D matrices that decellularize cadaveric tissue or a whole organ, followed by recellularization of patient-derived autologous cells. **(D)** Self-organizing morphogenesis, which relies on cell-cell interaction to spontaneously form the tissue-level structure and functional units.

necessary vasculature to initiate the repair process and exhibits limited chondrocyte proliferation. The transplant of autologous donor tissue harvested from a non-weight-bearing region to the damaged site may help. However, similar to the case with autologous skin grafts, this approach is subject to the availability of cells and donor sites. A cell-based treatment that harvests autologous chondrocytes and regrows them in culture before transplantation has also been tested, with the results showing prolonged improvement in excess of 10 years [18]. To enhance donor cell retention, scaffold-associated chondrocyte implantation has been used to provide temporary support until the scaffold can be replaced by a newly synthesized matrix, thereby creating relatively mature cartilage prior to transplantation [19]. Alternatively, allografts and cell-free substitutes was also researched and tested. One allograft substitute that takes advantage of chondrocytes' immunoprivileged status demon-

strated successful performance in relatively long-term treatment [20]. However, allografts and cell-free substitutes generally face a significant loss in viability after 10-15 years. In addition, the overall feasible size of cartilage grafts remains small owing to transport limitations, and their desired differentiation phenotype is also poorly understood.

Although a number of challenges lie ahead, skin and cartilage grafts can be considered successful examples of tissue engineering, and several have already been commercialized. Their success provided the initial framework for tissue engineering, that is, the culturing of cells in biocompatible material supplemented with biochemical and biophysical factors, and subsequently encouraged applications in many other types of tissue. However, the relatively simple strategy of combining cells and a matrix works for skin and cartilage because the vasculature is not an essential element in their

tissue function. The challenges are significantly greater when it comes to more complex tissues such as the foot processes of the podocytes in kidney glomeruli [21], radial vasculature of liver lobules [5], or the rotational alignment of cardiac muscle fibers in heart contraction [4]. The related engineering processes are thus likely to require more advanced technologies.

SYNTHETIC ACELLULAR SCAFFOLDS

The currently available engineering approaches are summarized in (Table 1). The early study of scaffolds focused on building acellular structures that would allow cell colonization, phenotype maintenance, and the delivery of biochemical factors or nutrients. Hence, porosity was the major focus. Solvent casting and particulate leaching are the most commonly used approaches to creating porous structures for cell attachment and nutrient penetration [22]. Specifically, a liquidized polymer is mixed with a water-soluble material such as salt. The 3D shape of the overall structure is achieved by direct molding, and porosity can be controlled by dissolving the salt particles in a water bath (Fig. (2)). However, owing to manufacturing limitations, the use of molded scaffolds is restricted to bulky structures of tens of millimeters to centimeters in scale [22]. Additionally, a 3D structure can be achieved by laminating porous, biodegradable two-dimensional (2D) sheets and assembling them layer by layer [23], and the resolution of lateral feature sizes can be improved using soft lithography. The process begins with fabrication of a polydimethylsiloxane (PDMS) mold. Then, by filling the mold with Poly(L-lactic-co-glycolic acid) (PLGA) using casting, spin-coating, or microfluidic molding, a laminated polymer sheet with a 2D pattern is achieved. Finally, a 3D structure is formed by fusing multiple layers of the PLGA membrane [24].

There are a number of other fabrication methods for synthesizing a porous scaffold, such as selective laser sintering, which uses local heat to fuse calcium phosphate particles [25]; fused deposition modeling, which uses a nozzle to deposit molten plastics in layers [26]; and electrospinning, which applies a high electric field to extract polymer fibers and weave them into 3D shapes [27]. In addition, to achieve patterns with better geometric control, photochemistry-based stereolithography uses light to initiate polymerization chain reaction, thereby causing solidification of the structure at the desired location [28].

It should be noted that the main purpose of using an acellular scaffold is to upgrade a conventional 2D cell culture into a 3D architecture. However, acellular scaffolds are produced through conventional manufacturing processes in mechanical or chemical engineering, and it is thus difficult to integrate them with living cells during the manufacturing process because UV exposure, heat energy, and/or solvent infusion may be harmful. Accordingly, one of the biggest challenges for acellular scaffolds is repopulating the cells after the fabrication process. The biodegradability and toxicity of the polymer after transplantation may also be issues in clinical applications. These challenges have greatly limited the progress of the acellular scaffold approach in recent years.

SYNTHETIC HYDROGEL CELL/SCAFFOLD CONSTRUCTS

Hydrogel use has become an increasingly popular approach in recent years. It allows the structural support and permeability of nutrients without concerns over biodegradability or toxicity. More importantly, instead of repopulating cells after scaffold fabrication, many hydrogel systems can accommodate the presence of cells during gelation [29, 30]. This capability allows the uniform distribution of cells throughout the hydrogel and also provides the 3D distribution of cellular adhesion sites. As the cells are allowed to spread, migrate, and proliferate in the hydrogel, as exemplified by mesenchymal stem cells [30] and neural cells [31], this approach may provide the best *in vivo*-like environment.

One study used the microfluidic channel as the molding template to pattern the collagen gel and shape the hydrogel into a specific geometry [32], although the tissue architecture was limited to the channel's 2D layout. Other researchers adopted photolithography owing to the flexibility of the optical patterns [33, 34]. When cells are present, the regions exposed to a low UV dosage undergo local polymerization. Then, after removal of the non-polymerized area, a rectangular region entrapped with cells is revealed. This method has been used to investigate the organization of cell elongation and alignment in response to the hydrogel boundary [33] and to construct a 3D hydrogel pattern by stacking multiple layers [34]. Inkjet printing is another potential approach to tissue fabrication. Similar to 3D printing, regular inkjet printing is used to dispense cells at chosen locations until a particular 3D form is achieved [35]. As inkjet printing is primarily applied to liquid solutions, one technical challenge is undesired spreading of newly deposited hydrogels. In addition, compatibility with living cells is also difficult to achieve. By precisely controlling the timing between gelation and dispensing, Pataky *et al.* recently demonstrated the possibility of printing a 3D hydrogel with such shapes as hollow tubes in which multiple cell types are entrapped and kept alive [36].

An alternative approach using microfluidic channels makes use of a new concept similar to a loom. Multiple hydrogel pre-polymer solutions with a biomolecular or cellular preload are organized as a planar stream. At the channel exit, the hydrogel with the pre-defined spatial composition is formed into a sheet via ion-diffusing cross-linking [37]. A complicated 3D structure can be formed by stacking the sheets or tubular structures or by rolling them into a translating capillary tube. Other methods that use cell sheets have been suggested, including combining micropatterning and gelation to produce a cell sheet with a heterogeneous cell co-culture [38-40] and using nanoscale grooves to align cardiomyocytes to form a cardiac tissue construct [41].

Although various engineering approaches have been proposed to fabricate hydrogels, their physical size remains small because of a lack of effective vasculature. One means of resolving this issue is to assemble arrays of microengineered hydrogels with doughnut shape into a 3D tubular construct that allows fluid perfusion through the interconnected lumens [42]. However, this is not a universal solution for all kinds of tissue constructs. Recently, an approach is to encapsulate networks based on carbohydrate-glass material inside a hydrogel block. After the carbohydrate-glass material is

Table 1. List of available methods for regenerating tissue with a specific shape.

Method	Application	Materials	Key Characteristics	Limitations	Ref.
Synthetic Acellular Scaffold					
Molding	Ear-shaped structure	PLGA	Simple mechanical molding and particulate leaching to control overall shape and porosity	Limited spatial resolution	[22]
Laminated foam	Nose-like scaffold	PLLA and PLGA	Stacking 2D porous membrane into complex 3D shape	Limited spatial resolution	[23]
Soft lithography	Multilayered PLGA grid	PLGA	Mold casting with improved lateral resolution at 10-30 μm	Requiring precise alignment of microscale features during layer assembly	[24]
Selective laser sintering	Bone implants	Calcium phosphate	Sintering calcium phosphate powder to precisely reproduce complex shapes of bone replacements	High temperature required; limited application to hard tissues such as bone	[25]
Fused deposition	Honeycomb-like interconnected network	PCL	Enables controlled porosity and complex structures such as regular geometrical honeycomb pores	High temperature required	[26]
Electrospinning	Nanofibrous scaffold for bone tissue	PCL	Nanoscale porosity from electrospun fiber mesh	Geometric shape difficult to control	[27]
Stereolithography	Bone ingrowth	PPF/DEF	Automated control of laser curing for geometric features with moderate resolution	Limited choice of materials; biocompatibility concerns	[28]
Synthetic hydrogel cell/scaffold construct					
Microfluidic molding	3D biopolymer matrix patterning	ECM gels	Microscale control of integrated cellular pattern and microenvironment; natural ECM used as the scaffold	Limited overall shape	[32]
Photolithography	Tissue construct with coherent cell alignment	Gelatin methacrylate	Controls cellular alignment and elongation of tissue formation	Alignment limited in 1D direction	[33]
	Photo-encapsulation of mammalian cells	PEG	UV patterning to define the geometry of a 3D construct	Cytotoxicity of UV exposure and photoinitiator	[34]
Inkjet printing	Hollow tube	Alginate	Dispensing of bioink droplets for stacking to produce macroscopic constructs	Requiring precise timing to balance gelation and spreading; limited overall shape	[36]
Microchannel	Mosaic hydrogel sheet	Alginate	Microchannel that controls biopolymer sheet flow and assembly of hydrogel sheets in 2D and 3D	Thickness limited to 150-350 μm ; continuous structures only	[37]
Cell sheet engineering	Myocardial tissue and hepatocyte culture	Gelatin	Micro-contact printing to arrange co-cultured cell sheets	Primarily for 2D patterns	[38]
Nano-grooves	Cardiac tissue	PEG hydrogel	Anisotropically nanofabricated substratum guides cell geometry, action potential velocity, and expression of cell-cell coupling protein	Requiring scaling up and implementation of nanoscale stimulus into a 3D tissue construct	[41]

(Table 1) contd....

Method	Application	Materials	Key Characteristics	Limitations	Ref.
Sequential assembly	Biomimetic vasculature	PEGDA	Sequential assembly of donut-shaped microgels into tubular structures with lumen	Manual assembly process for tubular structures that are hundreds of micrometers in scale	[42]
Casting	Vascular network embedded in 3D hydrogel construct	Agarose; alginate; PEG; fibrin; Matrigel	3D filament networks of carbohydrate glass for casting vascular networks; good biocompatibility	Minimum lumen size at scale of hundreds of micrometers	[43]
Decellularized matrices					
Detergent-based perfusion decellularization	Bone	Cow trabecular bone	Use of natural tissue or whole organ as scaffold; preservation of structural features with native features and resolution	Requiring cadaveric tissue or organ; difficult to repopulate appropriate cell types and restore functions	[47]
	Heart	Cadaveric rat hearts			[48, 49]
	Lung	Cadaveric rat lungs			[50-53]
	Kidney	Cadaveric rat kidneys			[54]
	Liver	Cadaveric mice, rats, ferrets, rabbits, and pigs			[55]
Self-organizing morphogenesis					
Self-organizing cell assembly	Periodic spots and labyrinth	Vascular mesenchymal cells in 2D culture	Spontaneous formation of labyrinthine pattern, which changes to periodic spots with addition of exogenous morphogens	Cell type dependent; control of 2D pattern alone demonstrated	[66]
	Periodic holes		Increased cell motility, leading to formation of periodic holes		[68]
	Radial and concentric rings		Use of microtechnology to guide the self-organizing system into desired forms		[69]
Pluripotent stem cells in self-organizing culture	Cortical tissue	ESCs in 3D aggregation culture	Recapitulation of embryonic corticogenesis and layer-specific neurogenesis with regional specification	Requiring precise timing control of mechanical and chemical stimuli crucial for correct <i>in vitro</i> development; clinical transplantation and integration of host tissue remaining in very early stages	[75],
	Intestine	Lgr5 stem cells in Matrigel	Generation of crypt-villus structures in the absence of a non-epithelial cellular niche		[76]
	3D eye-cup	ESCs in 3D Matrigel	Stepwise acquisition of optic cup, retina pigment epithelium, and neural retina tissue, as seen <i>in vivo</i>		[77, 78]
	Cerebral cortex	ESCs or iPSCs in 3D Matrigel	Production of mature cortical neuron subtypes, including cerebral organoids that recapitulate characteristic progenitor zone organization with outer radial glial stem cells		[79]
	Ear sensory epithelia	ESCs in 3D Matrigel	Sequential organization of non-neural, pre-placodal, and otic-placode-like epithelia, giving rise to hair cells bearing stereocilia bundles and a kinocilium with the functional properties of native mechanosensitive hair cells and sensory neurons		[80]
	Adenohypophysis	ESCs in 3D aggregate culture	Self-organizing into non-neural head ectoderm and hypothalamic neuroectoderm		[81]
	Liver tissue	Human iPSCs in Matrigel-coated dish	Generation of vascularized and functional human liver after transplantation		[82]

dissolved, the hollow lattice revealed can then serve as the perfusable vasculature. Importantly, the infusion of vascular endothelial cells into the vasculature allows the endothelial cells to line the vascular wall and form the endothelium. This approach may result in a new tissue regeneration concept in the future [43].

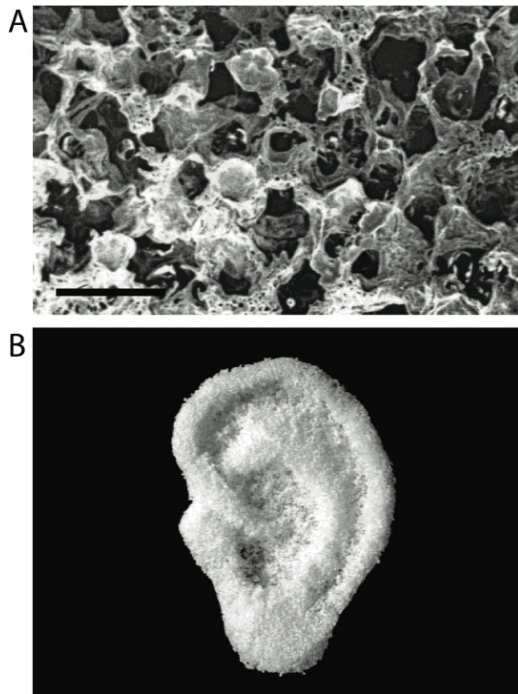


Fig. (2). 3D porous scaffold made by solvent casting/particulate leaching for PLGA. (A) Scanning electronic microscope image from 10% (w/v) PLGA/salt mixture; Scale bar, 500 μm . (B) Photographs of an ear-shaped PLGA porous scaffold (reprinted with permission from Ref. [22]).

DECELLULARIZED 3D MATRICES

As previously noted, the vascular network is an essential, and probably the most challenging, element in rebuilding tissue of a relatively large size. In reconstructing an entire organ, nutrient and gas exchange are critical not only for cell survival but also for organ function, and thus a new engineering approach is required. One possible solution is the use of a natural cadaveric organ. After the original cells from the cadaveric organ are decellularized using a detergent or ionic solution, the acellular natural scaffold with the original tissue microarchitecture and vascular network can be obtained as a template for tissue regeneration [44]. Decellularization was used fairly early on investigating cell functions and phenotypes in response to relatively simply structured matrices such as skin [17], adipose tissue [45], the mammary gland [46], and, more recently, bone [47]. The success of this early research encouraged the belief that decellularization is a suitable method for both simply structured tissues and complex organs with vascular networks. Combining perfusion-based decellularization with a recellularization process to repopulate the scaffold with autologous cells, whole organ engineering has been achieved in the heart [48, 49], lung [50-53], kidney [54], and liver [55], and has been tested in pre-clinical *in vivo* studies.

The combined decellularization/recellularization approach offers two major advantages. First, the removal of native cells may minimize the immune response. Second, when the natural scaffold material and geometric arrangement is retained, the approach is able to obtain the authentic microarchitecture, which is not possible using current fabrication technology. However, although the recent progress achieved in whole organ decellularization/recellularization suggests that the approach holds great promise, challenges remain before it can be tested in clinical trials. For example, issues surrounding the sources and availability of candidate donor organs of a similar size need to be resolved, an optimal decellularization protocol that retains the growth factors and bioactive molecules essential for cell activity must be identified, and, most importantly, an optimal recellularization protocol able to guide cells toward the desired location for recreating organ-specific structures and functions remains lacking.

SELF-ORGANIZING MORPHOGENESIS

Tissue development is a complex biological process that requires signal cascades of necessary components in a spatiotemporal pattern. Methods that attempt to rebuild tissue without an understanding of tissue development have proved ineffective [56]. In fact, such self-organizing processes as cell migration [8], proliferation [57], and cell-cell alignment [9] subsequent to cell settlement may defeat and frustrate such artificial attempts, eventually disorganizing the desired tissue layout and functions.

Does theoretical modeling offer any help? Self-organization is a common phenomenon in biomathematics whereby patterns spontaneously emerge both spatially and temporally. In Alan Turing's paradigm [58], periodic patterns spontaneously arise from the reaction and diffusion of chemical factors called morphogens. Gierer and Meinhardt modified the original Turing-type model, suggesting an underlying mechanism that requires only two key morphogens: a slowly diffusing activator produced by an autocatalytic reaction and a rapidly diffusing inhibitor produced in proportion to and able to inhibit that activator [59]. The general equations in the Gierer-Meinhardt model are as follows.

$$\begin{aligned} \frac{\partial u}{\partial t} &= D\nabla^2 u + \gamma \left[\frac{u^2}{v(1+ku^2)} - cu \right], \\ \frac{\partial v}{\partial t} &= \nabla^2 v + \gamma [u^2 - ev] \end{aligned} \quad (1)$$

where u and v refer to the slowly diffusing activator and its rapidly diffusing inhibitor, respectively; $D = D_u/D_v$ is the ratio of the diffusion coefficients of the activator and inhibitor; and γ is a scaling factor formulated by the domain size, biosynthetic timescale, and inhibitor diffusion coefficient. Activator production is modeled as an autocatalytic reaction that is down regulated by the presence of the inhibitor, whereas inhibitor production is modeled as proportional to the presence of activator. Production of both the activator and inhibitor is subject to first-order degradation quantified by c and e . Although only two key molecules are involved, the intensive interaction between them promotes highly ac-

tive self-organization in the system. With only a small degree of perturbation at the homogeneous spatial equilibrium in the initial condition, the interplay between activator and inhibitor creates patterns that are driven purely by the instabilities in the morphogenetic dynamics. These mathematical dynamics are able to create periodic patterns of “spots,” “labyrinths,” and “holes” as the concentration distribution of the morphogens (Fig. (3)). As such patterns are commonly seen in animal coats and skin pigmentation, this mechanism has been used to correlate many biological systems [60], including the pigment patterns on marine angelfish [61, 62] and zebrafish [63], hair follicle distribution [64, 65], and cell-cell assembly [66-69].

More specifically, the stripe formation observed in angelfish and zebrafish has been associated with the presence [61, 63] and chemotactic migration [62] of pigment cells in response to a chemical morphogen. Further, with regard to molecules, Jung *et al.* found a correlation between the reaction-diffusion mechanism and size, number, and distribution of appendages of feather germ formation [65]. In 2006, Sick *et al.* successfully identified the two molecules that determine hair follicle spacing: WNT for the activator and DKK for the inhibitor [64]. They calculated the change in inter-follicular spacing in response to the altered expression of WNT and DKK theoretically and verified it experimentally. As chemical morphogens are believed to establish the chemical pre-pattern of follicle development, this identification of morphogen molecules enabled further investigation of the detailed development process that takes place in response to a chemical pre-pattern [70]. More importantly, it demonstrated that Turing-type pattern formation is not simply a theoretical postulation, but exists in the physiological realm.

Turing-type morphogenesis can be applied to 3D tissue structures. For example, the 3D simulation of a labyrinth pattern yields the layered-sheet structure commonly observed in vessel branches and internal organ topology [71]. More complex patterns such as 2D branching structures can also be achieved using a higher degree model [72]. For example, Metzger *et al.* reported that lung airway branching can be classified as domain, orthogonal, or planar branching that occurs in sequence [73]. Developmental biologists have hypothetically attributed the phenomenon to modulated genetic programs, with a bifurcator responsible for branching and a rotator responsible for the rotation of the branching plane to achieve orthogonal branching. In applying Meinhardt model that expand the reaction-diffusion mechanism with the addition of a substrate chemical and a marker for

cell differentiation [72], Guo *et al.* found that a single set of partial differential equations (PDE) is sufficient to reproduce the cascades of all branching events for 3D branching morphogenesis [74]. Their results indicate that it may be more useful to consider the self-organization dynamics among a few interacting components than to view tissue development as the result of separate genetic modules. Understanding the dynamics of such variables as rapidly diffusing inhibitors and slowly diffusing activators using PDE may also help to identify their real physiological counterparts.

Compared to common engineering approaches that strive to rebuild tissue morphology using manufacturing technology, self-organization provides an alternative perspective, that is, spatial patterns may emerge spontaneously without the need for engineering specifications. Is it possible to combine the findings of developmental biology and biomathematics to advance tissue engineering? In 2004, it was discovered that, when homogeneously plated in culture, vascular mesenchymal cells spontaneously organize and aggregate into a multicellular structure in a periodic pattern similar to Turing-type morphogenesis [66]. A labyrinthine pattern was observed in normal culture conditions, and it was changed to a pattern of periodic spots with the addition of MGP [66] or a pattern of periodic holes with the inhibition of non-muscle myosin II [68]. This research showed a system capable of accommodating the change of molecular parameters, and the results are compared with theoretical simulations. Furthermore, with the aid of microtechnology, Chen *et al.* also identified a new phenomenon called cellular chirality, which is left-right-biased turning in cell migration [67]. The chirality accompanying cell migration eventually leads to macroscopic tissue formation with left-right asymmetry. In this series of experimental and theoretical studies, the beginnings of the integration of developmental biology, engineering, and biomathematics can be seen. The combined efforts of these multiple disciplines have created a new framework that works in concert with the self-organization process. With the assistance of theoretical predictions, a microtechnology-based engineering strategy was recently implemented to guide the direction of cell migration, thereby yielding the ultimate control of tissue formation, as exemplified by the radial and concentric rings shown in Fig. (4), which mimic the vasculature of liver lobules and the cross-section of compact bones [69].

Around similar period of time, a concept using a 3D culture for self-organizing tissue formation was introduced [75]. In contrast to conventional tissue engineering which focuses

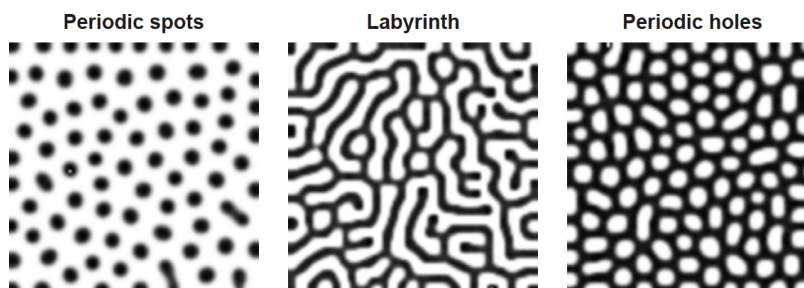


Fig. (3). Periodic patterns of “spots,” “labyrinths,” and “holes” as the concentration distribution of morphogens calculated using Turing’s reaction-diffusion mechanism.

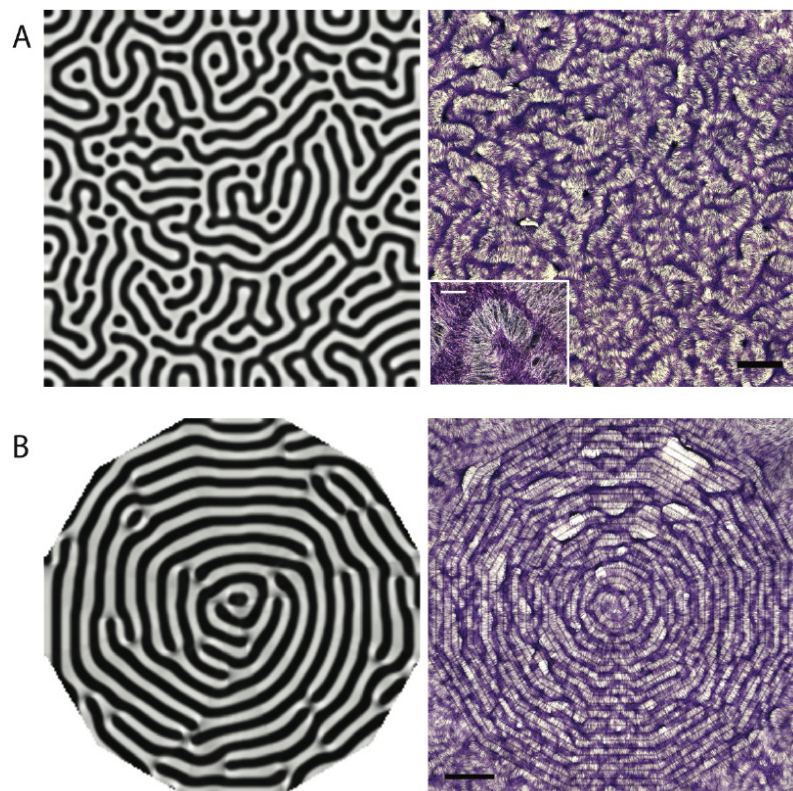


Fig. (4). Combination of biomathematics and engineering approaches to guide tissue formation via cell-cell assembly. **(A)** Numerical simulation and experimental results showing the labyrinthine pattern by the assembly of vascular mesenchymal cells in a petri dish; scale bar, 2 mm and 300 μm (inset). **(B)** With the aid of microtechnology, pattern formation can be predicted and guided into the desired morphology as concentric rings; scale bar, 2 mm (reprinted with permission from Ref. [69]).

on fabricating a scaffold with specific geometry, in this approach pluripotent stem cells are suspended in aggregation culture or embedded inside the hydrogel, thus allowing cell proliferation, migration, and eventual self-organization into a 3D structure without spatial guidance. Embryonic stem cells (ESCs) that undergo spontaneous re-aggregation in aggregation culture were used in these experiments where the enhanced cell-cell contacts in the 3D-like environment allowed the ESCs to form cortical neurons [75]. Another study focused on the culture of isolated single crypts in laminin-rich Matrigel, which allowed the construction of crypt-villus structures [76].

This approach has gained increased attention in recent years. With timed addition of chemical morphogenetic cues in aggregation culture or hydrogel, homogeneously distributed pluripotent stem cells were found capable of self-organizing into a 3D eye-cup [77, 78], cerebral cortex [79], inner ear sensory epithelia [80], and functional adenohypophysis [81]. The success of research in this direction indicates the possibility of replicating normal development *in vitro*. More importantly, it suggests the possibility of tissue formation with authentic microarchitecture such as layered retinal pigment epithelium, lens, and neural retina epithelium in eye-cup formation [77] and preplacodal and otic-placode-like epithelia with inner ear mechanosensitive hair cells in ear sensory epithelia [80]. The artificial fabrication of these spatial arrangements is impossible using current technology. In addition, this approach can also serve as an *in vitro* model for disease and organ development. For example, it has been

difficult to find an animal model capable of representing microcephaly, a neurodevelopmental disorder that results in a reduced brain size. However, using patient-derived induced pluripotent stem cells (iPSCs), the development of cerebral cortex in 3D gel was found to produce smaller neuroepithelial tissues and a large degree of neuronal outgrowth, which are reminiscent of the size features seen in patients with this condition [79]. Hence, this *in vitro* model of human brain development can recapitulate brain tissue features for both tissue regeneration and disease treatment. Finally, vascularized and functional liver tissue were recently achieved after transplantation using *in vitro*-developed liver buds formed by human iPSCs in aggregate culture [82], findings that hold promise for drug development and, potentially, clinical transplantation.

DISCUSSION AND FUTURE RESEARCH DIRECTIONS

This review provides a general overview of the development of tissue regeneration technologies from their beginnings to the most recent self-organizing morphogenesis in 2D and 3D formats. We have seen that early successes were achieved in relatively simple architectures such as skin and cartilage, which do not require an extensive vascular network or geometry. However, more complex functions require more complicated tissue architecture including heterogeneous cell arrangements and extensive vasculatures, which cannot be achieved by simply mixing cells with a supporting scaffold. Accordingly, a variety of engineering technologies

have been developed to modify the scaffold geometry as well as cell location, as summarized in (Table 1).

These achievements subsequently inspired the idea of “micromanaging” the tissue microenvironment with a complete set of artificially defined conditions, e.g., the spatio-temporal arrangement of cells, distribution of mixed chemical factors to induce levels of differentiation, and physical factors such as electric potential, stiffness, and porosity allowing cell migration and ECM deposition. Advances in micro- and nano-technologies may offer new opportunities for the implementation of morphogenetic cues. However, our current understanding and available tools remain limited in their ability to cope with such vast amounts of complex information. It is most likely impossible to micromanage, or dictate, cell-tissue interactions with precise analogy to natural circumstances. Furthermore, cells are not stationary units. Considering a biological network as a whole, its overall physiological function is the result of interactions among its constituent components such as cells, the ECM, and environmental factors. The organizing behavior such as cell alignment, migration, proliferation, and differentiation may subsequently disorganize artificial arrangements, eventually disrupting desired patterns and functions.

Therefore, it may be more appropriate to treat tissue formation as a problem of complex system. Rather than directly applying detailed instructions, working in concert with the process of self-organization may be more effective. As we have seen, the 3D culture of pluripotent stem cells with inducible factors is able to reproduce a variety of tissues such as eye-cup and cerebral cortex tissues. It should be noted that these reported tissue architectures are considerably more complex than those achieved by artificial approaches. They thus suggest a new concept that we need to take advantage of the self-organization process and only exert minimal engineering efforts to “guide” that process toward the desired outcome. For example, an engineering approach may be needed only to lay out the initial conditions, with the remainder of the tissue formation process left to self-organizing morphogenesis. The efficacy of such a strategy was recently demonstrated in works on controlling the pattern formation of vascular mesenchymal cells [68, 69]. Based on spontaneous cell-cell assembly, a small perturbation, i.e., an increase in cell motility, was shown to completely change the global pattern of multicellular structure from a labyrinth to periodic holes [68], thus suggesting the possibility of directing this self-organizing system toward a particular outcome without artificially arranging the cells into that desired layout. Furthermore, with the aid of micropatterning technology that defines the initial plating distribution of cells, such cell-cell assembly can be guided into more complicated patterns, e.g., rings and radial patterns, without detailed instructions [69]. Importantly, these methods are heavily reliant on self-organized cell alignment, migration, and aggregation. Working in collaboration with self-organization thus minimizes the amount of engineering effort required.

Additional theoretical work is needed to more effectively guide self-organizing systems and offer fresh perspectives on predicting the non-linear dynamics. Taking branching morphogenesis as an example, FGF10, BMP4, SSH, Sprty2, and MGP are known to be the essential morphogens in lung

airway formation. However, how their spatiotemporal interactions regulate the formation of airway branching remains unclear. The genetic approach, which completely knocks out specific genes, has been incapable to elucidate the interactive dynamics. Thus, when considering reproducing the branching morphogenesis *ex vivo*, implementation of these activities with precise time-varying changes in concentration and gradient becomes even more challenging. In PDE-based theoretical work [74], branching events are attributed to an activator-inhibitor pair. Tip bifurcation is the result of a time lag between the production and diffusion of the inhibitor and activator, and structural growth is the result of the activator’s migration toward the new substrate that serves as fuel for the reaction. These dynamics thus provide new insights for recapturing the complex interactivity. According to the theoretical modeling, to reproduce the dynamics of morphogenesis, the morphogen pair must be a slowly diffusing activator for short-range activation, e.g., BMP4, and a rapidly diffusing inhibitor for long-range inhibition, e.g., MGP. Thus, to implement such dynamics experimentally, a cell type that can produce both growth factors should be used. Also, a substrate, e.g., FGF10, should be homogeneously distributed in a 3D environment in order to serve as the fuel for activating the reactions. Thus, using an engineering approach to position a cluster of cells that proliferate and produce both activators and inhibitors in the 3D environment supplemented with FGF10 will ideally result in the cluster itself growing into a stalk and bifurcating into branched structures. Therefore, based on the guidance from theoretical prediction, the engineering effort required is reduced to setting up the initial condition with uniformly distributed FGF10 and positioning the cellular cluster, and there is no need to micromanage the subsequent development process.

In summary, the development of new solutions for tissue regeneration will require multidisciplinary collaboration among researchers in the areas of engineering, mathematical modeling, and developmental biology. Rather than micromanaging the interactions among numerous components, it is suggested that engineering efforts focus on setting up the initial conditions and/or global biochemical/physical factors during the intermediate process. With new insights likely to be revealed by developmental biology and mathematics, it is envisioned that future approaches will work primarily in concert with self-organizing morphogenesis, thereby guiding tissue formation toward more complex and desired forms for therapeutic purposes.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

ECM	=	Extracellular matrix
ESCs	=	Embryonic stem cells
iPSCs	=	Induced pluripotent stem cells

PLGA	= Poly(L-lactic-co-glycolic acid)
PLLA	= Poly(L-lactic acid)
PCL	= Poly(caprolactone)
PPF	= Poly(propylene fumarate)
DEF	= Diethyl fumarate
PEG	= Polyethylene glycol
PEGDA	= Polyethylene glycol diacrylate
SEM	= Scanning electronic microscope

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