Self-Organization and the Self-Assembling Process in Tissue Engineering

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Abstract

In recent years, the tissue engineering paradigm has shifted to include a new and growing subfield of scaffoldless techniques that generate self-organizing and self-assembling tissues. This review aims to cogently describe this relatively new research area, with special focus on applications toward clinical use and research models. Particular emphasis is placed on providing clear definitions of self-organization and the self-assembling process, as delineated from other scaffoldless techniques in tissue engineering and regenerative medicine. Significantly, during formation, self-organizing and self-assembling tissues display biological processes similar to those that occur in vivo. These processes help lead to the recapitulation of native tissue morphological structure and organization. Notably, functional properties of these engineered tissues, some of which are already in clinical trials, also approach native tissue values. This review endeavors to provide a cohesive summary of work in this field and to highlight the potential of self-organization and the self-assembling process for providing cogent solutions to currently intractable problems in tissue engineering.

Contents

1. INTRODUCTION AND MOTIVATION

Tissue engineering, developed by combining knowledge from molecular biology, materials science, biomechanics, and medicine, intends to produce tissue constructs to repair or replace native tissues compromised by trauma, pathology, or age. Central to this translational endeavor is the use of scaffoldless approaches that have allowed the fabrication of tissues for use in a plethora of applications, both clinical and experimental. The objective of this review is to describe advances in scaffoldless tissue engineering, concentrating primarily on self-organization and the self-assembling process. Clear delineations are provided between self-organization and the self-assembling process in tissue engineering, and limitations associated with the traditional scaffold-based tissue engineering paradigm are also presented. A description of the underlying mechanisms of these two important processes is provided, followed by a review of their application in various tissues and organs. Subsequently, the translational and clinical applications of self-organizing and self-assembling tissues are detailed, with particular emphasis on challenges to overcome before therapeutic use. Tissues engineered employing scaffoldless approaches can also be used for experimental applications, such as drug screening and injury models. As self-organization and the self-assembling process exhibit high potential for clinical translation and other uses, continued research in this area is clearly warranted.

1.1. Research in Traditional Tissue Engineering

The components of the traditional tissue engineering triad are cells, signals, and scaffolds. Cells, which are necessary to form tissues, need to reside in significant numbers within scaffolds. Primary cells are desirable for tissue engineering, as their phenotype is relatively unaltered, but isolation

of a large number of primary cells is challenging (1). Accordingly, tissue engineering strategies increasingly incorporate passaged cells, as well as adult, embryonic, or induced pluripotent stem cells because of their self-renewal capabilities (2). Signals or stimuli (e.g., mechanical, electrical, or biochemical) are also important in driving these cells to produce the tissues of interest. Different combinations of stimuli (e.g. bioreactors, growth factors) have not yet produced tissue with near-native properties, but recent work has identified additional beneficial stimuli for neotissue formation, such as enzymes and small signaling molecules (3, 4).

Scaffold development may be the most widely studied area of the traditional tissue engineering paradigm. Exogenous scaffolds are useful for providing structure to a developing tissue and allowing cells to adhere, proliferate, differentiate, and most importantly, secrete extracellular matrix (ECM) in a three-dimensional fashion. Because cells respond differently to substrates of different stiffness, manipulating scaffold stiffness can be a useful technique to control cell behavior (5). Scaffold porosity can be controlled to enhance cell infiltration (6). Also, it is important to note that for certain tissue engineering problems, scaffolds are necessary. For example, anchorage-dependent cells require attachment to a substrate for optimal viability and function (5, 7).

Tissue engineering approaches that use scaffolds are attractive for additional reasons. For example, in applications in which immediate load bearing is necessitated, the presence of an exogenous scaffold may provide the required mechanical integrity. Scaffolds also facilitate the tailoring of mechanical anisotropy. Furthermore, scaffolds afford the ability to precisely control the release of growth factors and other signals into the surrounding milieu. This potentially gives a biomedical engineer the ability to control the surrounding microenvironment long after construct implantation (8). Finally, scaffolds not only enjoy a shelf life that is typically greater than an engineered biological construct, but those without cells also face lower regulatory hurdles (9). Therefore, depending on the type of tissue being engineered and the approach to implantation, scaffold-based tissue engineering strategies may be preferable.

However, issues with scaffold-based tissue engineering hinder its use in certain applications. Scaffold degradation is rarely synchronized to neotissue formation, making remodeling and integration difficult, thus compromising functional properties. Furthermore, toxicity and immunogenicity due to scaffold creation, seeding, or degradation are of concern (10). The presence of a scaffold may also alter the phenotype of cells that come into contact with it (11). Finally, scaffolds may be directly detrimental, as they may physically obstruct mechanotransduction, for example via stress shielding (12). Although tissue engineering is still a young field, problems associated with the use of scaffolds, and strong desire for clinical and experimental application, have motivated research into alternate tissue engineering approaches.

1.2. Advantages of Scaffoldless Tissue Engineering Technologies

Over the past three decades, a spectrum of approaches in tissue engineering and regenerative medicine have been developed. In particular, various scaffoldless technologies, taking advantage of cells' natural ability to synthesize tissue and respond to signals, have also appeared. Scaffoldless tissue engineering approaches include traditional techniques, such as pellet (13) and aggregate culture (14); self-organization techniques, such as bioprinting and cell-sheet engineering; and the self-assembling process of articular cartilage and fibrocartilage. Thus, the term scaffoldless tissue engineering refers to any platform that does not require cell seeding or adherence within an exogenous, three-dimensional material.

Scaffoldless approaches demonstrate certain advantages over traditional scaffold-based approaches by overcoming limitations associated with the use of scaffolds (**Figure 1**). First, scaffoldless tissue engineering does not involve the exposure of cells to the harsh processing requirements

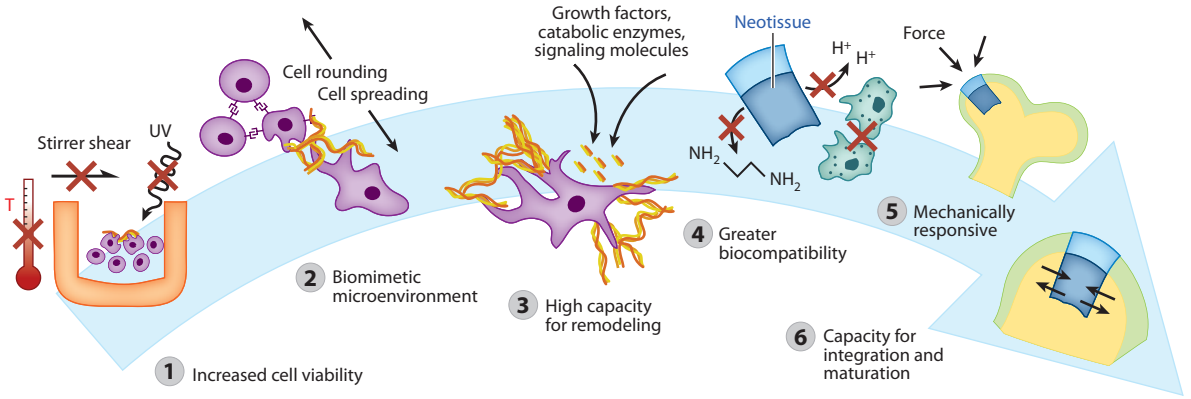

Figure 1

Scaffoldless tissue engineering displays significant advantages from construct formation to implantation of tissue.

of scaffold-based constructs (e.g. spinner shear, elevated temperatures, toxic polymerizing chemicals), a difference that leads to increased cell viability (15). During construct formation, scaffoldless tissue engineering provides a biomimetic microenvironment allowing for a high degree of cell-cell communication and the maintenance of cell phenotype, both of which can increase ECM production (16–19). Additionally, without an intervening scaffold, tissue synthesis and remodeling may occur more readily and without the need for scaffold degradation. After implantation, scaffoldless tissues release no toxic degradation by-products and hold no potential for scaffold-based immunogenicity (20). Once implanted in vivo, mechanotransduction occurs directly upon scaffoldless tissues, and thus stress shielding is avoided. Finally, the high cellularity and relative naiveté of scaffoldless tissues result in greater potential for integration and maturation after implantation of tissue constructs in vivo. Each of these advantages underscores an essential step in the process of tissue synthesis for clinical translation. Thus, scaffoldless technologies represent significant advances in tissue engineering, especially with regard to clinical applications.

1.3. Defining Self-Organization and the Self-Assembling Process in Tissue Engineering

The term self-assembly has been used to describe many distinct phenomena in science and engineering, including crystal growth, protein folding, and even galaxy formation (21, 22). The study of self-assembly is not formalized, and thus the definition for this topic may vary slightly across different fields (23). In general, however, self-assembly refers to systems in which order results from disorder in a spontaneous manner, that is, without the use of external energy or force (23).

Correspondingly, there is ambiguity surrounding the use of the terminology self-assembly, or self-assembling process, with regard to tissue engineering (24). The ultimate goal in tissue engineering is the recapitulation of the native tissue formation that generally occurs spontaneously in vivo through multiple biological processes (e.g. differential adhesion, tissue fusion). Thus, the development of a self-assembling process, or other technology resulting in spontaneous tissue formation, remains appealing to researchers. Confusingly, the tissue engineering literature has inconsistently applied the terms self-assembly and self-organization without definition (25–30). Despite fundamental differences among them, forms of aggregate culture, bioprinting, and other culture systems have all been dubbed self-assembly and self-organization. Therefore, this review defines these terms, specifically as they apply in tissue engineering (**Figure 2**). In order to clearly

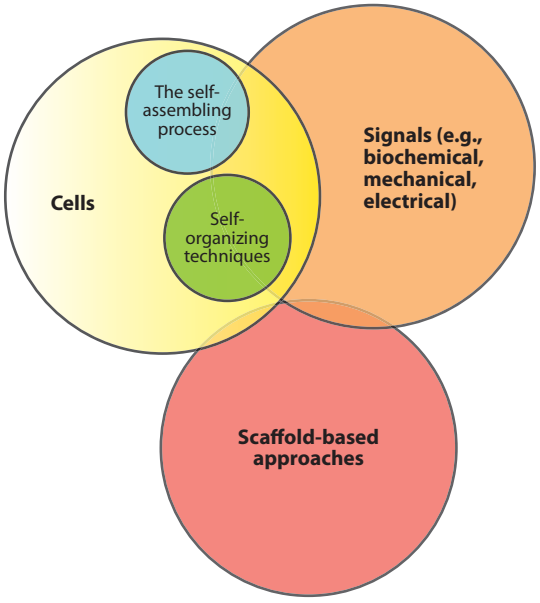

Figure 2

The self-assembling process and self-organization are subsets within scaffoldless tissue engineering.

define self-assembly and self-organization as they apply in tissue engineering, this review strives to understand the differentiating characteristics of various tissue engineering culture systems. It also elucidates the self-assembling process as a novel tissue engineering technique with respect to underlying biological processes and characteristics of self-assembling tissues.

To clarify the differences between self-organization and the self-assembling process in the relatively young field of tissue engineering, it is useful to consult existing distinctions between the two terms as used in established fields of study. In physics, chemistry, and biology, definitions of these terms are based on the field of thermodynamics, which states that self-organization describes a process in which order appears when external energy or forces are input into the system (31, 32). By contrast, for a self-assembling process, no external forces are required to promote order (23). Succinctly put, self-organization and the self-assembling process occur in open versus closed systems, respectively (23).

Thus, self-organization in tissue engineering can be defined as a subset of techniques within scaffoldless tissue engineering, which produce tissues that demonstrate organization upon the application of external forces. Self-organizing constructs can display gross morphology or structure recapitulative of native tissues. Self-organization techniques have the ability to produce engineered tissues of up to several centimeters, in the geometry of native tissues, as seen in cylindrically shaped tendon, bone, and nerve constructs (29, 33, 34). Additionally, self-organizing tissues can give rise to structures and/or regional variations found in native tissues, such as intima, media, and adventitia layer segregation in engineered blood vessels or optic cup and neurosensory tissue formation in engineered retina (35, 25).

With the above definition in mind, bioprinting and cell-sheet engineering can be categorized as examples of self-organization. These techniques use external forces, such as physical manipulation or thermal input, to direct cell position, after which cell-driven remodeling (e.g. tissue fusion, described in Section 2.1) occurs (28, 36). Bioprinting first places cells into a templated pattern

and then takes advantage of the ability of these cells to secrete ECM and fuse into a continuous tissue with the appropriate morphology (28). Similarly, in cell-sheet engineering, separate cell sheets are first seeded in monolayers and then detached with the use of heat. Afterward, these monolayers are stacked or rolled and undergo remodeling and fusion into patches or tubes of tissue with clinically relevant sizes (36). Underlying these examples are the biological mechanisms by which self-organization takes place, most notably the process of tissue fusion (see Section 2.1), which is also observed during tissue development in vivo. Furthermore, some self-organizing tissues possess appreciable functional properties, with values at times comparable to those of their native counterparts. Thus, the relevance and significance of self-organization techniques in tissue engineering are that highly biomimetic constructs, which are more easily translated toward applications, are produced.

By applying the characteristics of self-assembly in thermodynamics, the self-assembling process in tissue engineering can be defined as a scaffoldless technology that produces tissues that demonstrate spontaneous organization without external forces; this occurs via the minimization of free energy through cell-to-cell interactions. As in thermodynamics, the difference between the terms self-assembling process and self-organization in tissue engineering is whether external energy or forces are introduced into the system. Although both are subsets within scaffoldless tissue engineering, the self-assembling process is unique in that organization arises without the input of external forces (**Figure 2**). Self-assembling tissues possess the following specific characteristics: (*a*) the use of a nonadherent substrate to minimize tissue free energy, (*b*) a sequential set of phases that recapitulate native tissue formation, (*c*) tissue constructs with sufficient size and morphology to be clinically relevant, and (*d*) functional properties with values comparable to those of native tissue. Notably, self-assembling tissues follow the differential adhesion hypothesis, a fundamental mechanism of developmental biology. Thus, self-assembling tissues are highly biomimetic and comprise promising candidates for clinical application.

2. MECHANISMS OF SELF-ORGANIZATION AND THE SELF-ASSEMBLING PROCESS

Native tissue formation and maturation are complex processes that often take months or years of coordinated signaling. One challenge in in vitro tissue engineering is that some of the natural biological processes driving in vivo tissue formation have been absent. Prominent among these is differential adhesion, which is responsible for the successful formation of a variety of tissues during morphogenesis (37). Thus, it is in the interest of tissue engineers to pursue technologies that more directly replicate native tissue development. The self-assembling process and self-organization both exhibit fundamental biological processes that naturally occur in vivo (**Figure 3**).

2.1. Tissue Fusion in Self-Organizing Tissues

Tissue fusion comprises a series of events in developmental biology that are involved, for example, in neural tube formation, skeletal patterning, and cardiovascular development (38). Tissue fusion has been defined as the process by which two or more isolated cell populations make contact and adhere (38). This includes cell-to-cell contact and/or cell-to-matrix contact of two previously separated cell populations. In addition, tissue fusion includes matrix-to-matrix contact and ECM remodeling.

Interestingly, a biological process similar to tissue fusion also occurs in self-organizing and self-assembling tissues (**Figure 3*a***). For example, self-organization approaches in engineering musculoskeletal, cardiovascular, neurosensory, and digestive tissues all display a fusion process in

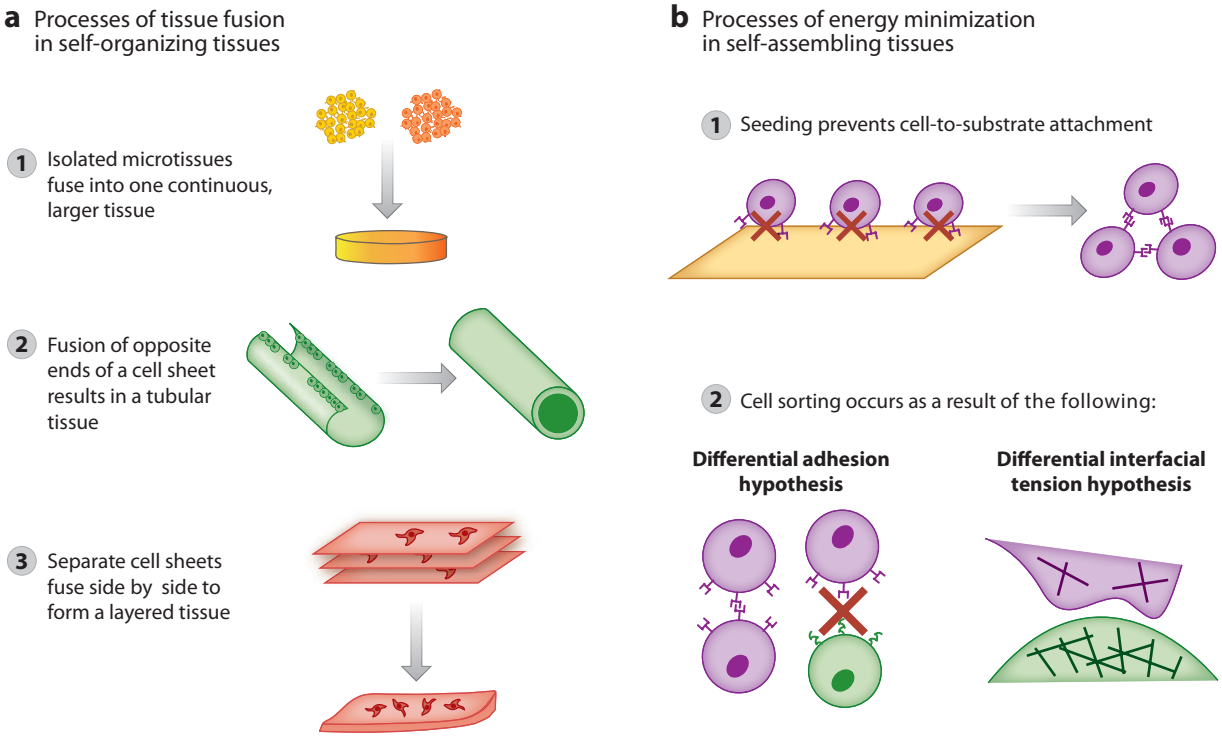

Figure 3

Self-organizing tissues, as well as self-assembling tissues, undergo the process of tissue fusion by various means (*a*). Additionally, self-assembling tissues follow the principle of energy minimization via cell-to-cell interaction (*b*).

which previously isolated cells and/or ECM converge into a continuous whole (25, 29–33, 39, 40). Self-organizing optic and liver tissues are engineered such that micromasses fuse into a continuous neotissue (25, 30). Self-organizing nerve, bone, tendon, and ligament tissues all rearrange into cylindrical tubes following seeding, concomitant with tissue fusion of opposite ends of an initial cell sheet (29, 33, 39, 40). Bioprinting and cell-sheet engineering, as self-organization technologies, also display tissue fusion. Bioprinted tissue solutions fuse together over time to form sheets, tubes, or other intended morphological features, after deposition onto a substrate (41). Similarly, cell-sheet engineering of corneal, cardiac, and vascular tissues involves the fusion of distinct layers of ECM to form one continuous tissue (35, 42, 43). These processes may occur once the cell sheets or bioprinted solutions are directed into contact with one another by the use of external forces. Finally, self-assembling cartilage and fibrocartilage also display tissue fusion, as separate cells eventually converge into a continuous tissue (44, 45). Therefore, self-organizing tissues and self-assembling tissues both exhibit a process reminiscent of the tissue fusion found in native tissue development.

2.2. Energy Minimization During the Self-Assembling Process

In various instances and stages of tissue development, cells interact to minimize the overall free energy of the tissue they comprise, resulting in several phenomena including cell sorting. When biologists observed the sorting behavior of dissociated germ layer cells, models to describe

this behavior began to be formulated. The most successful of these is the differential adhesion hypothesis, which states that a tissue will tend to minimize the adhesive free energy of its cell populations via cell-to-cell binding (37, 46). Accordingly, a mass of cells may be conceptualized as a liquid that works to minimize its surface tension (known as tissue surface tension). Tissue surface tension will determine whether these cells sort to the center or periphery when mixed with another cell population to form a heterogeneous tissue, with cells from the tissue of higher surface tension maximizing their intercellular adhesion and, thus, being enveloped (47). The cell-to-cell adhesion molecules thought to be primarily responsible for differential adhesion are cadherins, which are calcium-dependent transmembrane proteins. Indeed, tissue surface tension has been shown to be linearly correlated to the number of cadherin molecules present, although theoretically any cell-to-cell adhesion molecule may drive differential adhesion (37).

Although the differential adhesion hypothesis is the most widely accepted model of these phenomena, recently, another explanation, the differential interfacial tension hypothesis, has been gaining recognition (26, 48–51). This explanation also conceptualizes tissue as a liquid that acts to reduce its surface tension, but it highlights the possibility that forces generated by cellular components such as the membrane and cytoskeleton may dictate cell sorting. The differential adhesion hypothesis and the differential interfacial tension hypothesis may be related. The underlying driving tendency of cells in a tissue to minimize their free energy does not change between these theories (49). Recent work has brought these two hypotheses together, showing that induced germ layer cells display differential binding affinities as well as different cell cortical tensions (48). Alongside of this, it has also been reported that intracellular cytoskeletal reorganization can occur as a result of cadherin-mediated adhesion (52) and that tissue surface tensions measured from actomyosin contractility outweigh those generated by cadherin interactions (53, 54). Thus, it is reasonable to speculate that energy minimization in a developing tissue may be due to initial cadherin interactions leading to downstream signaling and cytoskeleton reorganization, resulting in cell aggregation and sorting (55).

The self-assembling process works by the principle of free energy minimization (**Figure 3*b***). During the self-assembling process, cells are seeded upon a nonadhesive surface (44, 45). This prevents cell attachment and thus compels cells in a developing neotissue to spontaneously adhere to one another in order to minimize free energy. Consequently, immunohistochemical staining, at 1 day and 4 days after cell seeding, displays extensive N-cadherin-mediated cell-to-cell binding, which occurs without the influence of external forces (56). This mimics the process of mesenchymal condensation, in which N-cadherin levels increase dramatically prior to chondrogenesis (57). Accordingly, the first characteristic of the self-assembling process is the use of a nonadherent mold to minimize construct free energy. Energy minimization will also lead to cell sorting, and indeed, groups utilizing the self-assembling process to investigate cell sorting have seen the segregation of endothelial cells and fibroblasts seeded in agarose wells (26). Furthermore, this sorting mechanism and the self-assembling process have also been seen to depend on the cytoskeleton (58–60). Therefore, differential interfacial tension may also be relevant to this culture system, as interfacial tensions are generated in part by the cytoskeleton (49, 50, 61). In summary, the self-assembling process in tissue engineering follows the principle of free energy minimization without external forces, leading to the recapitulation of mechanisms relevant to native tissue development.

3. FUNCTIONAL PROPERTIES OF SELF-ORGANIZING AND SELF-ASSEMBLING TISSUES

The techniques involved in self-organization and the self-assembling process vary by tissue and also within tissues, but all display promising results for tissue engineering (**Table 1**). As

Table 1 Functional properties of tissue constructs engineered by self-organization and the self-assembling process

Tissue/organ	Tissue engineering method	Properties attained	Translational status	Key References
Vasculature	Self-organization by cell sheet engineering	Average burst pressure of 3,490 mm Hg (465 kPa)	Phase I clinical studies	L'Heureux et al. 1998 (35), Gauvin et al. 2010 (72), Mironov & Kasyanov 2009 (73), Gwyther et al. 2011 (75), McAllister et al. 2009 (77), Haraguchi et al. 2012 (78), L'Heureux et al. 2007 (105)
	Bioprinting	Engineered vascular tube of 900 μm diameter with 300 μm wall thickness	In vitro studies	Norotte et al. 2009 (41)
Articular cartilage	Self-assembling process	~3-mm thick constructs with compressive aggregate modulus of 280 kPa; tensile stiffness at 2 MPa	Preclinical animal studies	Responte et al. 2012 (4), Hu & Athanasiou 2006 (44), Elder & Athanasiou 2009 (99)
	Pellet culture	~1-mm spherical construct	In vitro studies	Zhang et al. 2004 (13)
	Aggregate culture	~500-μm spherical construct	In vitro studies	Furukawa et al. 2003 (14)
Meniscus	Self-assembling process	Compressive instantaneous modulus of up to 800 kPa and tensile stiffness of up to 3 MPa (tensile modulus in circumferential and radial directions of up to 3 MPa and 1.5 MPa, respectively)	Preclinical animal studies	Hoben et al. 2007 (45), Aufderheide & Athanasiou 2007 (74), Huey & Athanasiou 2011 (96), Huey & Athanasiou 2011 (97)
Eye	Self-organization	Transparent tissue of 55-μm thickness	Preclinical studies	Eiraku et al. 2011 (25), Nishida et al. 2004 (36), Proulx et al. 2010 (42), Zhang et al. 2011 (85), Nishida et al. 2004 (110)
Tendon and ligament	Self-organization	Tangent modulus of 15 to 17 MPa	Preclinical animal studies	Calve et al. 2004 (33), Hairfield-Stein et al. 2007 (63), Huang et al. 2005 (64), Calve et al. 2010 (66)
Liver	Self-organization	Albumin production; prolonged secretion of the oxidation enzyme cytochrome P450; production of α1-antitrypsin	In vitro studies	Tzanakakis et al. 2001 (59), Koide et al. 1990 (86), Landry et al. 1985 (88), Hansen et al. 1998 (90), Ohashi et al. 2007 (91)
Nerve	Self-organization	Conduction velocities of 12.5 m/s	In vitro studies	Baltich et al. 2010 (29), Adams et al. 2012 (82)

intrinsically scaffoldless platforms, self-organization and the self-assembling process hold a variety of advantages (as detailed in Section 1.2 above). Several of these, such as a biomimetic microenvironment and the ability to respond to mechanical signals, aid in the development of neotissue functional properties. Consequently, self-organizing and self-assembling tissues have been assessed for various functional properties such as mechanical strength, electrical conductivity, and biochemical secretion rate.

3.1. Self-Organization in Tissue Engineering

As explained above, self-organization in tissue engineering refers to engineered tissues that exhibit spontaneous generation of distinct structures or gross morphology reminiscent of native tissues without exogenous scaffolds. It is distinct from the self-assembling process in that external manipulation occurs (e.g., bioprinting of cells to their appropriate positions, thermal variation to detach a cell sheet). Partly owing to the advantages conveyed by scaffoldless tissue engineering, substantial functional properties have been reported with many self-organizing tissues. Self-organization in tissue engineering has been used, through several different methods, to engineer a wide variety of tissues from various systems of the body (e.g., the musculoskeletal, cardiovascular, neurosensory, and digestive systems).

Several tissues from the musculoskeletal system have been engineered using approaches that display self-organization. In general, self-organization in musculoskeletal tissues such as bone, tendon, ligament, and skeletal muscle starts with monolayer culture of cells in protein-coated (e.g. laminin) Sylgard plates with external anchors. These anchors are used to exert tensile forces on the seeded monolayer as it contracts and rolls up, leading to the formation of a cylindrically shaped tissue (33, 39, 62–64). Expression of cadherins and other adhesion molecules during self-organization of musculoskeletal tissues is uncharacterized and open to further research. Additionally, the role of the various coatings on self-organization is unclear; it is possible that coating degradation throughout culture exposes the initial monolayer to a nonadherent surface. Thus, it is conceivable that minimization of free energy will occur through the use of a nonadherent surface, but this needs to be examined.

Self-organizing musculoskeletal tissues display morphological and structural features, as well as some mechanical functionality, reminiscent of those in corresponding native tissues. In self-organizing bone, localization of osteocytes in lacunae, formation of lumen-containing structures similar to blood vessels, and development of cellular areas similar to bone marrow all occur (39, 65). Self-organizing tendons and ligaments exhibit collagen fiber alignment reminiscent of that in native tissues (66). Self-organizing skeletal muscle displays myoblast fusion into myotubes and the formation of muscle-specific structures (e.g. hexagonal architecture and Z lines) (64). Additionally, both fast-twitch and slow-twitch muscle subtypes, with relative relaxation and contraction rates, have been engineered (67).

Engineered musculoskeletal tissues also maintain functional properties. For instance, self-organizing ligaments and tendons exhibit tangent modulus values of 15 to 17 MPa, concomitant with abundant collagen I and III staining (33, 63). Similarly, self-organizing muscle has been reported to exert a specific force of up to 140 N/mm^2, which is within the range displayed by native tissue, during culture and stimulation within an electrical bioreactor (68). Self-organizing bone with tangent modulus of up to 29 MPa and compressive strength surpassing 1.5 MPa after 6 weeks of culture has also been reported (39, 69). By comparison, scaffoldless bone culture on an orbital shaker (i.e., aggregate culture) does not form large macroscopic tissue and thus is not mechanically testable (70). However, mechanical properties of native bone can be much greater, with compressive stiffness reaching hundreds of MPa and compressive strength in the range of tens

of MPa (71). Therefore, self-organizing musculoskeletal tissues demonstrate promising results, especially with regard to tissue morphology and functional properties, and more research should be conducted to elucidate the potential of these technologies.

Cardiovascular tissue engineering has benefitted from self-organization in tissue engineering, especially in the synthesis of vascular constructs, where a paradigm shift from polymeric scaffold-based to cell-based scaffoldless techniques has occurred over the past decade (27). Self-organizing vasculature has been demonstrated via the use of cell-sheet engineering, in which high confluence monolayers are harvested as a whole sheet, and external forces are then introduced by rolling the cell sheet on a mandrel (35, 72). During culture, these initially separate layers spontaneously fuse to form a tube structure (35). Self-organizing vasculature mimics the layered organization of native blood vessels, with an inner endothelial lining, a medial smooth muscle cell layer, and an outer adventitia rich in ECM (73). These self-organizing constructs can reach tensile moduli of up to 2 MPa and are capable of withstanding burst pressures of up to 465 kPa (73). Another reported technique uses high-density seeding of smooth muscle cells in annular agarose wells, similar to the ring-shaped mold used in self-assembling meniscus-shaped fibrocartilage (74–76). External forces are then introduced when these tissues are manually aligned, after which they fuse into vascular tubes, which display tensile strengths and moduli of up to 500 kPa and 2 MPa, respectively (75). Because of their functional properties, these vascular constructs have great clinical potential, and currently some of them are in clinical trials (77).

In cardiac tissue engineering, one technique stacks multiple layers of cardiac muscle sheets together, before this construct self-organizes into a continuous tissue (78). Another example is bioprinting, which utilizes layer-by-layer deposition of cells onto a substrate to place cell aggregates in close proximity, eventually leading to tissue fusion (79). Self-organizing cardiac muscle displays native tissue structures, electrical conductivity, contraction rates similar to native tissues, and the ability to continuously contract without fatigue (78, 80, 81). Although self-organizing cardiac muscle and vasculature display significant similarities to native tissue, developmental phases during construct formation, if any, are not characterized. Additionally, the use of external forces in engineering of self-organizing cardiovascular tissues makes them distinct from the self-assembling process in tissue engineering. Owing to its high potential for clinical translation and simple manufacturing procedures, self-organization may be a suitable platform to solve problems associated with cardiovascular tissues.

Self-organizing neurosensory tissues, such as optic cup, cornea, and nerve, display structural features or functional properties similar to those of native tissues. Akin to tendon, self-organizing nerve starts with monolayer culture of tendon fibroblasts on laminin-coated Sylgard plates, with subsequent seeding of neural cells. This culture then contracts and fuses into a tube-shaped tissue around two anchors, with an inner nerve cell layer and an outer fibroblast layer (29). It has been shown that self-organizing nerve has conduction velocities (12.5 m/s) comparable to those of rat neonatal sciatic nerve (29). Furthermore, these self-organizing constructs have also been cocultured in association with glial-like cells differentiated from adipose-derived stem cells, although conduction velocities were not measured (82). Complex self-organizing optic tissues have also been engineered. In these, formation of a distinct optic cup is followed by segregation from stratified neurosensory tissue (25). Self-organizing cornea is another example, in which corneal epithelial cells, limbal epithelial cells, and corneal fibroblasts have been used to recreate the layered structure of native cornea (83). Seeding of corneal endothelial and epithelial cells on each side of a self-organizing fibroblast layer leads to tissue fusion (42). Although self-organizing optic tissues show promise, especially with regard to the recapitulation of native tissue organization, little work has been done to assess tissue functional properties. To compare, other scaffoldless cultures of optic retina and cornea using rotational culture and centrifugation, respectively, have

also been pursued, but no functional properties have been characterized (84, 85). Therefore, future research in neurosensory engineering should focus on the quantification of tissue function.

The liver, as part of the digestive system, has been the topic of numerous tissue engineering approaches. Self-organization of liver tissues results in biochemical secretion of several functional enzymes, as well as native structural organization. This self-organization technique involves initial seeding of hepatocytes on a surface coated with adherent proteins (e.g., collagen or glycoproteins), which leads to the hepatocytes self-organizing into spheroid structures after several hours or days (30, 86). As this method uses an adherent coating for cell attachment, it is categorized as self-organization. Self-organizing liver tissues can reach up to 2.5 mm in diameter, and it has been shown that the size of these spheroids is linearly correlated with initial cell seeding concentration (87). Self-organizing liver tissues also possess several features of developing tissues, such as bile canalicular formation, abundant cell-to-cell communication, cuboidal hepatocyte morphology, and cell sorting (88–90). Additionally, self-organizing liver tissues secrete several functional proteins. Albumin secretion rates are equivalent to those of freshly isolated hepatocytes, and prolonged secretion of cytochrome P450 oxidation enzymes has also been reported (30). It has also been demonstrated that, on a per cell basis, self-organizing liver tissues can produce more α1-antitrypsin than individual hepatocytes can and that urea and bile excretion into canaliculi occurs (91–93). Self-organizing liver tissues display a large variety of biochemical functions, and future research should investigate and enhance their translational potential.

Self-organization in tissue engineering is not synonymous with the self-assembling process in tissue engineering. In contrast to the self-assembling process, self-organization often requires external forces, manipulation, or seeding on an adherent surface. Continued research on self-organizing tissues, especially focused on the basic mechanisms by which these tissues form, is needed.

3.2. The Self-Assembling Process in Tissue Engineering

As explained earlier, the self-assembling process is separate from self-organization in tissue engineering. The self-assembling process for articular cartilage is a good example with which to describe the self-assembling process in tissue engineering because it has been reported to follow the characteristics of self-assembling tissues (**Figure 4**) (56). The first two characteristics of these tissues are (*a*) the ability to minimize free energy without the use of external forces and (*b*) a distinct set of phases reminiscent of those in native tissue development. The first phase in development

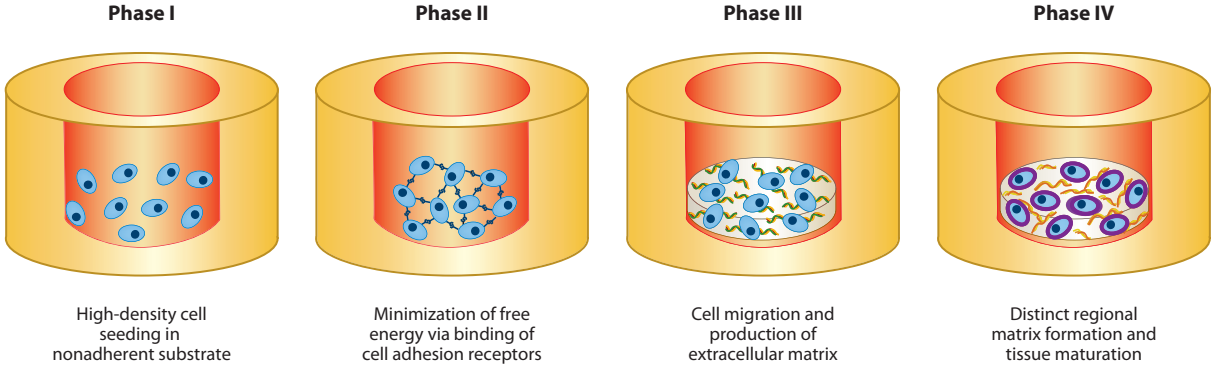

Figure 4

Different phases in the development of self-assembling articular cartilage.

of self-assembling articular cartilage is high-density seeding of articular chondrocytes in a nonadherent agarose well. The nonadherent agarose well used here prevents substrate adhesion and promotes the minimization of free energy via cell-to-cell interactions in the second phase (44). Instead of agarose, 2-hydroxyethyl methacrylate (94) and semipermeable membranes (95) have also been used as nonadherent substrates. In the second phase, the seeded articular chondrocytes express high levels of N-cadherin, which mediates cell-to-cell adhesion and tissue fusion, resulting in neotissue formation without the use of exogenous forces (56). In the third phase, collagen VI is produced throughout the self-assembling tissue, and high levels of the glycosaminoglycan (GAG) chondroitin-6-sulphate are synthesized. ECM maturation is the final vital step in cartilage formation, and self-assembling articular cartilage mimics this aspect of native tissue development. In the fourth phase, collagen VI localizes to the pericellular matrix as production of collagen II increases, and relative levels of chondroitin-6-sulphate and chondroitin-4-sulphate change to reproduce those seen during native cartilage development (56). Because collagens and GAGs confer tensile and compressive properties to native tissue, appropriate levels of these ECM components are tantamount to achieving adequate functional properties in engineered tissues.

Fibrocartilages such as the knee meniscus and temporomandibular joint (TMJ) disc are tissues whose shape is specific to their mechanical functions. The self-assembling process allows tissues to be grown in anatomically correct shapes. For fibrocartilage, analogous to self-assembling articular cartilage, high-density cocultures of chondrocytes and fibrochondrocytes can be seeded in ring-shaped nonadherent agarose molds to form anatomically shaped menisci that maintain the wedge profile of native menisci (96). The generation of native tissue anisotropy is also possible with self-assembling tissue. For example, self-assembling fibrocartilage in an anatomically shaped mold displays a tensile modulus four times higher in the circumferential direction than in the radial direction (74). Moreover, self-assembling fibrocartilage displays mechanical properties on par with or approaching those of native tissue, with compressive instantaneous modulus values of up to 800 kPa and tensile stiffnesses of up to 3 MPa (96, 97). Similarly, in self-assembling articular cartilage, near-native levels of aggregate modulus and tensile stiffness (of 280 kPa and up to 2 MPa, respectively) have been attained (4, 98, 99). Self-assembling tissue may also be grown to clinically relevant sizes. Articular cartilage up to 6 mm in diameter and 3 mm in thickness and fibrocartilage up to 13 mm in diameter and 1.5 mm in thickness have been reported with this process (4, 96). Thus, the self-assembling process of articular cartilage and fibrocartilage represent significant advances in the emerging area of scaffoldless tissue engineering.

4. PROGRESS TOWARD CLINICAL APPLICATION OF SELF-ORGANIZING AND SELF-ASSEMBLING TISSUES

The ultimate focus of tissue engineering is to achieve clinical application of engineered tissues (e.g., cartilage, vasculature) and systems (e.g., musculoskeletal system, cardiovascular system). To reach clinical translation, engineered constructs should be easy to manufacture, preferably in a cost-effective fashion. They must also be able to integrate with native tissue and to remodel themselves accordingly. Additionally, they should match native tissue biomechanical and biochemical properties, in concordance with the recipient's age and immune system. Currently, several self-organizing and self-assembling tissues are in preclinical studies as well as clinical trials (**Figure 5**).

4.1. Preclinical Models

Self-organization and the self-assembling process in tissue engineering have shown promising outcomes in preclinical animal studies, which are fundamental to clinical translation.

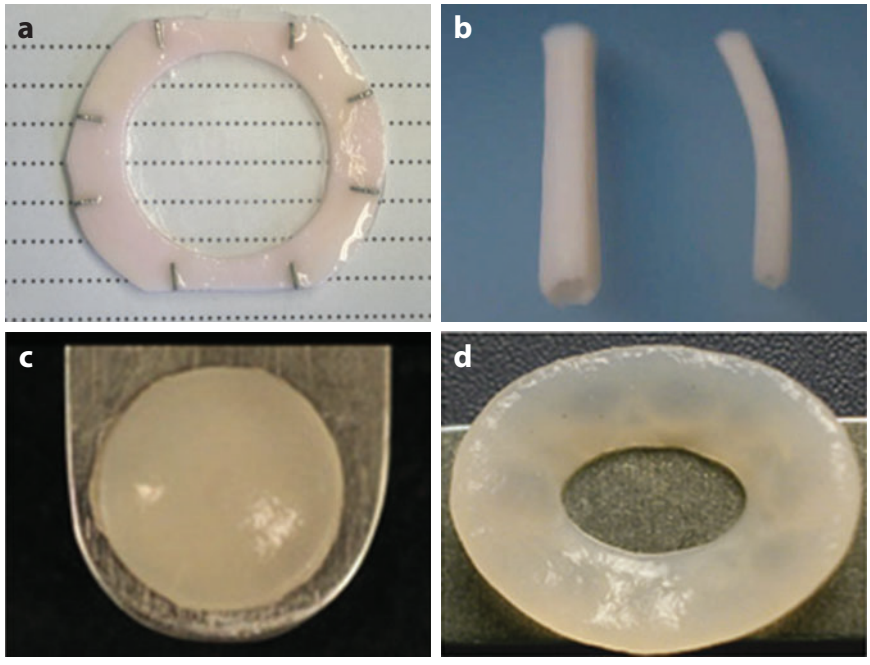

Figure 5

Clinically relevant–sized constructs engineered by self-organization and the self-assembling process include (*a*) self-organizing cornea, (*b*) self-organizing vasculature, (*c*) self-assembling articular cartilage, and (*d*) self-assembling meniscus. Images reproduced with permission from References (*a*) 42, (*b*) 41, (*c*) 4, and (*d*) 96.

One example of this is in tissues such as tendons and ligaments, which, owing to their interfacial nature, have been the focus of research toward achieving integration between different tissue types—for example, tendon with muscle and ligament with bone. Because of the lack of an intervening scaffold, self-organizing tendon and ligament may have greater capacity to integrate with other tissues (100). For instance, 1 or 2 months of in vivo implantation of self-organizing bone-ligament-bone constructs in rat medial collateral ligament (MCL) defects shows appreciable gross and histological integration of bone to native bone and of ligament to native ligament, as well as greater mechanical integrity between ligament and bone (100). Similarly, 4-week implantation of self-organizing tendon in rat tibialis anterior tendon defects leads to tissue integration and mechanical properties on par with those of neonatal rat tibialis anterior tendons (66). Self-organizing tendon-muscle integration has also been accomplished, with these interfacial constructs withstanding loading at physiological strains (101, 102). Therefore, one advantage of self-organizing and self-assembling tissues is the ability to interface with other tissues.

Self-organizing and self-assembling tissues display maturation in the form of ECM production and organization, which is crucial for tissue function. Self-assembling articular cartilage implanted subcutaneously for 4 weeks in mice results in tissue maturation and enhanced tensile and compressive stiffness (4). It is believed that the high cellularity of self-assembling cartilage enables the neotissue to rapidly remodel and increase tensile and biochemical properties in vivo. Similarly, implanted self-organizing ligament also exhibits collagen alignment in the direction of native tissue, as well as elastin organization as seen in native ligaments (66). Additionally, following implantation, an average twofold increase in construct thickness and cross-sectional area was observed (100). Akin to ligament, implanted self-organizing tendon results in increases in collagen content

and fibril diameter (66). Passaged chondrocytes from goats have been used to create constructs in custom bioreactors, under conditions that may fall under the umbrella of self-assembling articular cartilage (95). Implanted constructs were secured under periosteal flaps or adhesives. Without the periosteal flaps, most constructs became loose and were lost within the first month. However, for those that remained, after 2 months, tissues in the defects appeared hyaline-like (95). Further characterizations of mechanical and frictional properties for these constructs will provide additional evidence of their clinical usability. In general, self-organizing and self-assembling tissues demonstrate exciting potential in preclinical models, especially with regard to integration and maturation. However, further studies are necessary to explore these advantages and reach clinical translation.

4.2. Clinical Trials

Self-organizing vascular constructs are one of the most promising scaffoldless engineered tissues currently in preclinical and clinical studies. Initial successes with in vitro studies on cell-sheet engineering of human vascular tissue led to the development of self-organizing vascular constructs (27). These grafts are formed from age- and risk-matched human cells within a donor age range of 57 to 65, which in some cases reduces the risk of immunoglobulin-related immune response after implantation in a patient (77). In addition, these constructs recapitulate native vascular morphology and biological responses to relaxation and contraction stimuli, such as cyclic AMP and calcium ions (103, 104). In clinical trials with a high-risk human population (end-stage renal disease patients), patency rates of self-organizing grafts have been shown to be high, at 78% (1 month) and 60% (6 months) post implantation (77). Furthermore, preliminary assessments of vessel compliance reveal a beneficial 2.7–4.8-fold increase in some patients, indicating increased tissue elasticity after implantation (105). These grafts can also undergo devitalization and freezing, followed by rehydration, for later implantation into patients (106). This greatly increases their potential shelf life and thus clinical applicability. Because of these successes with early clinical application, self-organizing vasculature represents a model technology for clinical translation of tissue engineering.

Aside from vascular constructs, self-organizing cornea using a detachable cell sheet grown on a thermally responsive hydrogel (107) has also shown promising results in human trials (108). Implantation of self-organizing corneas in patients suffering from Salzmann's nodular degeneration led to significant improvements in gross transparency and visual acuity, as reported by patients (108). In corneal tissue engineering, epithelial-stromal interactions are important for the long-term survival of constructs in vivo (109). Accordingly, these self-organizing corneal constructs display an intrinsic ability to adhere to corneal stroma, representing an added clinical advantage, obviating the need for sutures during implantation. Furthermore, these constructs recapitulate native apical-basal cell and matrix organization (110). Despite these successes, however, further clinical trials and long-term follow-up studies are needed to explore the long-term safety and efficacy of these engineered tissues.

5. USE OF SELF-ORGANIZING AND SELF-ASSEMBLING TISSUES IN RESEARCH MODELS

In addition to clinical applications, self-organizing and self-assembling tissues have also been used as in vitro models to screen drugs and to study injury and disease. Producing a tissue model that duplicates in vivo human conditions represents a major challenge. Self-organizing and self-assembling tissues have the advantage of recapitulating the morphological structure and organization of native tissues. Furthermore, some of these tissues also mimic fundamental biological

processes seen in vivo. Therefore, self-organization and the self-assembling process are promising tools to study tissue formation, behavior, and trauma.

Injury models, including wound healing in skin and cornea, as well as liver damage, represent one cogent application of self-organizing tissues. In vitro models of wound healing are limited by the absence of multiple epithelial cell layers and a lack of epithelial-mesenchymal interactions, characteristics that are both present in vivo (111). Accordingly, because of its multilayered structure, engineered cornea is a promising candidate for studying corneal wound healing (112). Indeed, the process of wound healing in self-organizing corneas is reminiscent of human cornea reepithelialization, displaying wavelike epithelial cell migration as well as laminin V and collagen VII production (113). Furthermore, accelerated wound healing after treatment with epidermal growth factor (EGF) and fibrin, as occurs with native tissue, was observed in these models (112). Similarly, liver spheroids have been used as in vitro models to screen drugs for liver injury (59). For example, cytochalasin D treatment affects spheroid formation and hepatocyte morphology in these tissues, resulting in fivefold lower albumin secretion. Therefore, self-organizing constructs can be used to study tissue injury.

Disease models can also be investigated using self-organizing and self-assembling tissues. For example, cardiac hypertrophy, which is a consequence of increased biomechanical stress, often leads to pathological conditions (114). To study this complex disease in vitro requires the recapitulation of structural and functional features of native cardiac tissues. Recently, self-organizing cardiac tissues have been used as in vitro tools to study the hypertrophic phenotype (115). Self-organizing cardiac tissue subjected to biomechanical stress in vitro successfully demonstrates structural remodeling, increased secretion of clinical hypertrophy markers such as atrial natriuretic peptide (ANP), and electrophysiological changes, as found in cardiac hypertrophy in vivo. Furthermore, clinically used pharmacological antihypertrophic treatments partially reversed this hypertrophic behavior, akin to what is observed in vivo (115). Though a relatively new technology, self-organizing tissues represent a platform technology that can be employed to provide in vitro analogs of in vivo disease conditions. Thus, further exploration of self-organizing and self-assembling tissues as research models may represent a promising area for future research.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Self-organization in tissue engineering can be defined as a subset of techniques within scaffoldless tissue engineering that produce tissues that demonstrate spontaneous organization. The self-assembling process in tissue engineering is a separate subset within scaffoldless tissue engineering, defined as a technology that produces tissues that demonstrate spontaneous organization from the minimization of free energy through cell-to-cell interactions and without external forces.

Self-organization and the self-assembling process are promising tissue engineering approaches that have already shown great potential for engineering complex tissues with functional property values approaching those of native tissue. Of particular significance is the fact that some self-organizing tissues have already been used in clinical applications. For example, self-organizing engineered vascular constructs have been used in hemodialysis patients (35, 77, 104, 105), and self-organizing cornea constructs have achieved beneficial results in patients suffering from Salzmann's noduluar degeneration (85, 108, 110). In addition to this, self-organized hepatocyte spheroids have been used in pharmacological screening of drugs (59). Furthermore, self-assembling cartilage recapitulates sequential phases of development seen in native cartilage formation (44, 56). These recent key findings pave the way for engineering more complex tissues with greater biochemical and mechanical properties, and they encourage future research to enhance self-organization and the self-assembling process for wider use.

Future directions for research on self-organizing and self-assembling tissues should concentrate on achieving clinical application. Before this can occur, however, certain basic characteristics of these processes need to be obtained. Cell source needs are an especially important issue for self-organizing and self-assembling tissues, as these techniques are highly cell intensive. To alleviate this, cocultures including primary cells and stem cells should be explored (116, 117). Formation of complex tissues or structures using cell mixtures or cocultures brings forth additional issues. For instance, mixtures of cells, each expressing different adhesion molecules, need to be studied to understand what drives pattern formation and eventual fabrication of biomimetic structures. These adhesion molecules are often transient and dynamic, and, thus, computational models need to take this into account (118). Additionally, to understand the role of cytoskeleton contraction in self-assembling tissue, cell cortical tension and tissue surface tension should be quantified. This is made more difficult by the fact that cadherin-mediated cell-to-cell interaction influences cell cortical tension. These basic characterizations must be carried out to help identify suitable stimuli and conditions that can be employed in tissue engineering approaches using the self-assembling process and self-organization.

In general, tissue engineers should also continue to expand their repertoire of signals and stimuli. To date, tissue engineering studies have focused predominantly on the use of growth factors to enhance tissue. In the future, specialized bioreactors, and novel signaling agents such as catabolic enzymes, may lead to improvements in tissue functional properties (119). Similarly, continued identification of beneficial combinations of stimuli for self-organizing and self-assembling tissues is necessary (29, 30, 120, 121). Finally, the use of nondefined media components in tissue culture should be reduced. For instance, fetal bovine serum (FBS) often contains high concentrations of immunoglobulin and may trigger immune responses in human trials (122). To solve this, serum-free culture can eliminate such immunoglobulin-related immune response (123). Despite the relative dearth of basic characterization on the self-assembling process and self-organization, the results thus far suggest that these processes can be employed in promising manners to fabricate some of the most complex tissues and structures of the body, including articular cartilage, fibrocartilage, bone, tendon, ligament, vasculature, cardiac muscle, liver, nerve, and cornea.

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