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TOPICAL REVIEW

Tissue engineering by self-assembly and bio-printing of living cells

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Abstract
Biofabrication of living structures with desired topology and functionality requires the interdisciplinary effort of practitioners of the physical, life and engineering sciences. Such efforts are being undertaken in many laboratories around the world. Numerous approaches are pursued, such as those based on the use of natural or artificial scaffolds, decellularized cadaveric extracellular matrices and, most lately, bioprinting. To be successful in this endeavor, it is crucial to provide *in vitro* micro-environmental clues for the cells resembling those in the organism. Therefore, scaffolds, populated with differentiated cells or stem cells, of increasing complexity and sophistication are being fabricated. However, no matter how sophisticated scaffolds are, they can cause problems stemming from their degradation, eliciting immunogenic reactions and other a priori unforeseen complications. It is also being realized that ultimately the best approach might be to rely on the self-assembly and self-organizing properties of cells and tissues and the innate regenerative capability of the organism itself, not just simply prepare tissue and organ structures *in vitro* followed by their implantation. Here we briefly review the different strategies for the fabrication of three-dimensional biological structures, in particular bioprinting. We detail a fully biological, scaffoldless, print-based engineering approach that uses self-assembling multicellular units as bio-ink particles and employs early developmental morphogenetic principles, such as cell sorting and tissue fusion.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Self-assembly is the autonomous organization of components, from an initial state into a final pattern or structure without external intervention [1–4]. Living organisms, in particular the developing embryo, are quintessential self-organizing systems. Histogenesis and organogenesis are examples of self-assembly processes, in which, through cell–cell and cell–extracellular matrix (ECM) interactions, the developing organism and its parts gradually acquire their final shape. Ultimately, the success of engineering and fabricating functional living structures will depend on understanding the principles of cellular self-assembly and our ability to employ them. This fact is being gradually recognized across the tissue-engineering community, as except for a few successes [5–7] the field is yet to present viable solutions to the growing demand for novel regenerative technologies. Thus, future

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biofabrication approaches (including but not restricted to the field of tissue engineering) aimed at re-establishing the functionality of damaged tissues and organs will need to focus on mobilizing developmental morphogenetic processes coupled with requirements of adult biology, in short, the body's innate regenerative capability [8–10]. We will need to understand questions such as why the salamander can re-grow its limb but we cannot [11], or why the liver is the only internal human organ capable of regeneration [12]. This is important because though there is always a hope that *in vitro* tissue-engineering efforts will eventually lead to the long awaited breakthrough, it is also possible that the multileveled and evolutionary established nature of cells and organisms will continue to defeat this hope.

In this review we overview recent progress in fabricating living structures of definite shape and functionality by implementing developmental principles and processes, and describe a rapid prototyping technology that is based on the bio-printing of self-assembling multicellular building blocks.

2. Fundamentals of tissue engineering

Tissue engineering has emerged as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of tissue substitutes [13, 14]. The fundamental goal of tissue engineering is to regenerate or replace defective, diseased or missing tissues and organs. Examples of engineered grafts that are currently under pre-clinical studies include engineered skin, cartilage, bone, blood vessels, skeletal muscle, bladder, trachea and myocardium [9, 15–19]. Three 'classical' tissue-engineering approaches include [20] (i) use of an instructive environment (e.g. bioactive material) to recruit and guide host cells to regenerate a tissue, (ii) delivery of repair cells and/or bioactive factors into the damaged area and (iii) cultivation of cells on a biomaterial scaffold in a culture system (bioreactor), under conditions designed to engineer a functional tissue for implantation. In all cases ((i) and (ii) *in vivo*, (iii) *in vitro*), the engineered environments are designed to direct the cells—added from exogenous sources or mobilized from the host—to regenerate a specific tissue structure and function [9, 21–23]. When a tissue is engineered *in vitro*, cells (the actual 'tissue engineers') are placed into a biomaterial scaffold (providing the structural and logistic template for tissue formation) and cultured in a bioreactor (providing microenvironmental control and the necessary molecular and physical regulatory signals). Once a desired developmental stage is achieved (in most cases measured by critical functional properties), the tissue construct is implanted into the host, where further maturation and integration are anticipated.

At this time, tissue engineering opens several exciting possibilities: (i) to create functional grafts suitable for implantation and repair of failing tissues, (ii) to study stem cell behavior and developmental processes in the context of controllable three-dimensional (3D) models of engineered tissues and (iii) to utilize engineered tissues as models for studies of physiology and disease [22–27].

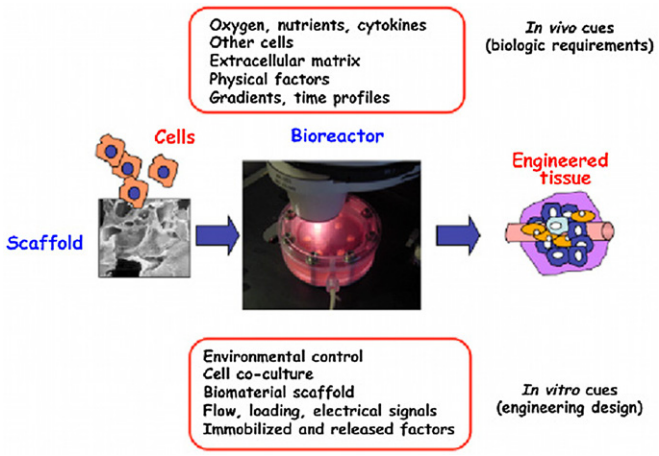

Figure 1. Biomimetic paradigm. *In vivo*, the progression of tissue development and remodeling depends on the interaction of time and space gradients of multiple factors that are not entirely known. The 'biomimetic' approach to tissue engineering aims to utilize these same factors through the design of biomaterial scaffolds (providing structural, mechanical and logistic templates for cell attachment and tissue formation) and bioreactors (providing environmental control, exchange of nutrients and metabolites, and the molecular and physical regulatory signals). This way, the biological requirements inspire the design of tissue-engineering systems, whereas tissue engineering provides controllable models of high fidelity for regenerative biology studies. (Reproduced from [2] with permission of Elsevier.)

In what follows, we briefly review some fundamentals of classical tissue engineering and present examples of self-assembly-based approaches.

2.1. Biomimetic approach to tissue engineering

In living organisms, tissue development is orchestrated by numerous regulatory factors, dynamically interacting at multiple levels, in space and time. Recent developments in the field of tissue engineering are aimed at designing a new generation of tissue-engineering systems with an *in vivo* like, but fully controllable cell environment. Such a 'biomimetic' environment, as a result of biology and engineering interacting at multiple levels [23, 26], should be suitable to direct the cells to differentiate at the right time, in the right place and into the right phenotype and eventually to assemble functional tissues by using biologically derived design requirements (figure 1). Thus, these environments must be able to provide the cells with the same regulatory factors—molecular, structural, and physical—that govern the *in vivo* cellular processes [21, 24, 27], and thereby unlock their full potential for tissue development and regeneration. Progress in the environmental control of tissue growth and maturation in bioreactors, directing cell fate using soluble clues (e.g. morphogens), mechanical or electrical stimulation, are increasingly orienting the field towards biologically inspired designs with a real-time insight [9, 27].

2.2. Cell sources

The choice of repair cells is central to any of the many different modalities of tissue engineering—injection of repair

cells (with or without biomaterial), implantation of a fully formed cell-based graft or mobilization of the host cells into the site of injury [20, 23, 26, 28]. One obvious requirement is that of immune tolerance of the repair cells. Utilization of autologous adult stem cells (i.e. cells obtained from the patient) has the advantage of being patient-specific and alleviating immune rejection or transmission of disease. While adult stem cells were first isolated from the bone marrow, recent literature supports their presence in a variety of extramedullary organs including adipose tissue, dental pulp, circulating blood, amniotic fluid and joint synovium [29–33].

In general, adult human mesenchymal stem cells have documented capacity to form a number of tissues (including cartilage, bone, fat and blood vessels), but not necessarily cardiac muscle, nerves, hepatocytes or Langerhans islets. Embryonic-like stem cells, including the newly derived induced pluripotent cells (iPS) [34, 35], have essentially unlimited potential for expansion *in vitro* and differentiation in multiple cell lineages. Recent exciting developments in iPS cells from various human tissues opens the possibility of creating autologous embryonic-like cells for cardiac regeneration, with the caveat that the derivation of iPS cells involves, at least at this time, genetic transformation. It is expected, however, that the use of small molecules will gradually and completely replace gene transfer in the derivation protocols of patient-specific adult human cells. We propose that the 'conditioning' of stem cells—both adult and embryonic-like—by biophysical stimulation in 3D culture settings may result in the stabilization and maturation of differentiated cell phenotypes.

Several promising paths toward deriving the 'right' cells for the repair of a range of human tissues and the right technology to utilize their potential need to be pursued until one or more of these options is translated into clinical practice.

2.3. Tissue-engineering scaffolds

Tissue replacements are developed by culturing cells on three-dimensional scaffolds to develop functionality and then implanted *in vivo*. Depending on what tissue is to be replaced, the properties of scaffolds will vary along several parameters, including biological substances used, porosity, elasticity, stiffness and specific anatomical shapes. To make a scaffold, biomaterials are processed into 3D architectures suitable for cell seeding and cultivation.

The choice of biomaterial is generally guided by the need to restore tissue-specific structure and cell signaling, and to match the necessary physical behavior (such as load-bearing or signal propagation). Scaffolds also need to serve as 'logistic templates' by providing the cells with specific topological features (from nano to micro and macro scale), mechanical environment (that cells can sense at multiple levels), surface ligands and the ability to release cytokines. Some of the novel scaffold designs enable an active and dynamic interplay with the cells [21]. As the cells deposit their own extracellular matrix, the scaffold-biomaterial is expected to fully degrade and a tissue-like structure to form and progressively integrate within the surrounding host tissue upon implantation. This approach has been implemented in the reconstruction of various tissues, including skin, bone, cartilage, meniscus and bladder, and has, in some cases, been further translated to clinical practice [5, 36–39].

Scaffold-based tissue engineering faces some challenges [40, 41], including (i) immunogenicity, (ii) acute and long-term inflammatory response resulting from the host response to the scaffold and its biodegradation products, (iii) mechanical mismatch with the surrounding tissue, (iv) difficulties in incorporating high numbers of cells uniformly distributed within the scaffold and (v) limitations in introducing multiple cell types with positional specificity. These difficulties, along with the increased understanding of developmental and morphogenetic processes, have led many groups toward the development of 'self-assembly' approaches in which individual cells organize into multicellular subunits (e.g. spheroids, sheets or cylinders) [42–45]. The individual subunits further arrange themselves into larger tissue structures with little intervention and in most cases without the use of exogenous scaffolds [46, 47].

2.4. Scaffold-free tissue engineering

Functional small-caliber arteries were engineered by cell cultivation on biodegradable fibrous polyglycolic acid (PGA) scaffolds in a bioreactor with dynamic pulsatile flow [48]. Collagen deposition and alignment during construct maturation is crucial for achieving adequate mechanical strength (e.g. burst pressure) needed for implantation. Maturation of vascular constructs under cyclic radial strain allowed for circumferential assembly and alignment of collagen in a helicoidal pattern similar to native blood vessels [49]. However, the use of biodegradable scaffold led to the residual presence of polymer fragments disrupting the normal organization of the vascular wall [50]. For such a mechanically demanding application, balancing the degradation rate of the scaffold material with tissue remodeling in the patient remains a challenge.

In parallel, self-assembly (in particular, scaffold-free) approaches demonstrate that fully biological tissues can be engineered with specific compositions and shapes, by exploiting cell–cell adhesion and the ability of cultured cells to grow their own ECM, and thereby help reduce and mediate inflammatory responses. One of the impressive examples of self-assembly methods is the sheet-based tissue-engineering technology developed by L'Heureux and colleagues [43]. Using this approach, sheets of human smooth muscle cells (SMC) and fibroblasts were grown on culture plates in the presence of ascorbic acid to enhance collagen production, detached and wrapped around a porous, tubular mandrel to form the equivalents of media and adventitia of blood vessels. After several weeks of maturation in a bioreactor, lumens of the constructs were seeded with endothelial cells (EC). The resulting structures displayed strong mechanical properties, well-defined multilayer organization and an abundant, organized ECM. Autologous small-diameter vascular grafts engineered using this method are currently in clinical trials for hemodialysis access [44].

Okano and colleagues have developed a similar 'self-assembled' sheet based approach for cardiac tissue engineering. In this approach, neonatal rat cardiomyocytes were cultured on temperature-responsive culture dishes [51–53]. After harvest, electrical coupling of layered cardiomyocyte sheets occurred quickly through functional gap junction formation [54], and subsequent implantation in subcutaneous position demonstrated that pulsatile, layered cardiomyocyte sheets survived and grew for a prolonged period of time [55]. The versatility of this method has made it a good candidate to create functional and transplantable tissue sheets obtained from various cell types including epidermal keratinocytes [56], kidney epithelial cells [57] and periodontal ligaments [58, 59]. It has already been used in a clinical trial involving corneal transplantation, which promoted the recovery of weakened vision [60].

2.5. *Need for functional vascularization*

One of the most critical present problems in the field of tissue engineering is to provide vascular supply to thick constructs [61, 62], as molecular diffusion can assure the exchange of nutrients and oxygen only within an approximately ~100 μm thick layer of viable tissue [63, 64]. The provision of vascular conduits that have been engineered *in vitro* to pre-vascularize tissues [65–68] is a remarkable but not sufficient advancement, as the immediate connection to blood flow upon implantation still remains a problem.

For one application, self-assembly of cells into sheets provided an indirect solution to this problem [69] by sequential implantation of multiple cardiac sheets. Because the thickness of a single cardiac sheet (<80 μm) was smaller than the diffusional penetration depth of oxygen, the host vasculature was given time (1–3 days) to vascularize each individual transplanted sheet before the next one was added during the following surgery. This way, 1 mm thick vascularized myocardium was obtained. While achieving the goal of functional vascularization of thick tissue-engineered constructs *in vivo*, this method is clearly not applicable to patients due to the medical risk of multiple surgical procedures.

Another approach enabled the generation of fully viable, thick and functional cardiac tissue constructs *in vitro* using microchanneled scaffolds and bioreactor perfusion systems [70, 71]. The functional assembly of engineered cardiac muscle *in vitro* was enhanced by oxygen supply provided by mechanisms resembling those in normal vascularized tissues. To mimic the capillary network, cardiomyocytes and fibroblasts isolated from neonatal rat hearts were cultured on a highly porous elastomer with a parallel array of channels that were perfused with culture medium. To mimic oxygen supply by hemoglobin, culture medium was supplemented with a perfluorocarbon (PFC) emulsion. The structural and functional properties of the constructs were markedly improved, in a manner correlated to the improved supply of oxygen to the cells. It was postulated that the channel arrays can also serve as precursors for the formation of the vascular network.

However, the transition and integration of a tissue from an *in vitro* to an *in vivo* setting still needs to be addressed. From a surgical point of view, a tissue-engineered graft would contain a hierarchical macro- to micro-vascular tree ending with a connectable artery and vein so that perfusion of the whole graft could be immediately restored upon implantation. In order to fabricate such a construct, three gradual components of the vascular tree have to be formed: the capillary network (~10–20 μm, by induction of sprouting, co-culture or by cytokines), intermediate microvessels (50–500 μm, by microfabrication) and the macrovasculature (up to 2 mm, by tissue engineering). Capillary networks can form in co-cultures of osteoblasts or cardiomyocytes with endothelial cells, by natural cell assembly in either sheet [65, 72] or spheroid [73, 74] cell culture systems. Despite major improvements allowing design of microvascular networks *in vitro* using microfluidic technologies [75], pre-vascularized tissues face the challenge of functional anastomosis to the host vasculature. Recent clinical trials with engineered blood vessels using scaffold-based [7] and sheet-based methods [76] illustrate that engineering of the macro-vasculature, including the connectable artery and vein for surgical anastomosis, seems to be within reach. It is hoped that these vessels will exhibit mechanical competence (e.g. burst pressure, compliance, suturability) and antithrombogenic properties [77, 78]. However, it remains unclear how can the macrovasculature be connected to the capillary tree and perfused with blood.

2.6. *Self-assembly of vascular networks*

Self-assembly approaches may have major impact on the development of the intermediate vasculature, which is the 'missing link' for establishing a perfused vascular tree. Sefton and colleagues have reported a method based on the modular assembly of endothelialized microtissues to form macrotissues [79]. In this approach, modular tissue-engineered constructs were assembled from sub-millimeter-sized cylindrical modules of collagen or gelatin seeded with cells, and endothelialized at the surfaces [79, 80]. EC-covered modules then randomly self-assembled into a modular construct with interstitial spaces that enabled perfusion with medium or whole blood. It remains to be seen how stable is the self-assembled microvasculature for the long term and how it can integrate components enabling direct anastomosis to the host vasculature *in vivo*. In a similar fashion, several groups, including ours, are currently working on the fusion of endothelialized spheroids as means for creating microvessels *in vitro* [81–83].

To develop tissue-engineered constructs of clinically relevant size with a fully functional vasculature that can eventually be anastomosed to the host vasculature *in vivo*, we suggest a self-assembly approach shown in figure 2.

A network of macrovessels, including a connectable artery and vein, would be build (by bioprinting, see below), and then matured using a perfusion bioreactor to achieve mechanical properties necessary for implantation. Parenchymal and endothelial cells would be co-cultured to produce micro-vascularized units (in the form of cylindrical or spherical multicellular aggregates), sized to be below the diffusion

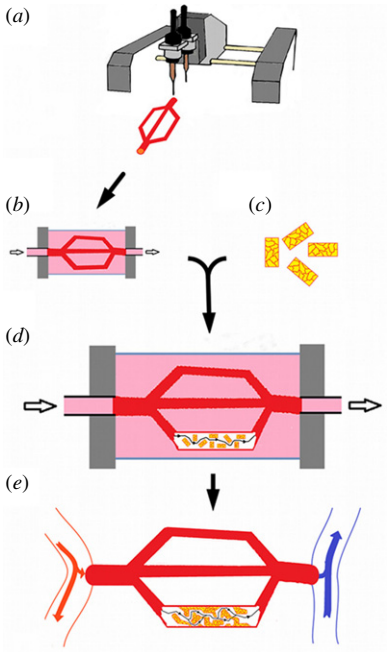

Figure 2. Model for thick construct tissue engineering using a self-assembly approach. (*a*) A macrovascular network (from a few mm to less than 1 mm in diameter) is built using bioprinting, then matured for several weeks in a perfusion bioreactor (*b*), in order to achieve adequate mechanical properties needed for future implantation in the host. (*c*) Micro-vascularized multicellular cylindrical modules (200 μm) are obtained from coaggregation of endothelial cells and the cell type of interest (ex: hepatocytes), and their surface is endothelialized. (*d*) EC-covered modules are then randomly assembled into a modular construct within each lumen of the macrovascular network (only one shown explicitly), and form interconnected channels (intermediate microvasculature: 50–500 μm) under medium perfusion. (*e*) The entire construct is then surgically connected to the host vasculature in an arterio-venous position.

limitation. After the surfaces are endothelialized, pre-vascularized units would be packed into the lumen of the matured macro-vessels and perfused to promote self-assembly of intermediate microvessels and their connection to a capillary network, to enable implantation by direct anastomosis to the host vasculature.

Cellular self-assembly approaches represent an alternative and offer a complement to scaffold-based tissue engineering. They allow establishing high cell density, controlled deposition of extracellular matrix and positional specificity of cell patterning. Translation of cell and tissue self-assembly approaches into the clinical field will necessarily depend on our ability to understand the principles underlying such approaches.

In what follows, we present examples of early developmental self-assembly processes that will be utilized for the 'biofabrication-by-bioprinting' technology.

3. Developmental mechanisms of cellular self-assembly

It is through cellular self-assembly that the morphologically featureless zygote evolves into the fully developed organism with its numerous structures of widely varied shapes and forms. Although the sequence of morphogenetic processes that underlies early development is under strict genetic control, additional physical mechanisms are mobilized to move mass and make shapes. In turn, the changes brought about by physical processes (e.g. diffusion, changes in shape, molecular and ion concentrations) provide feedback to gene expression. The interplay of genetic and physical processes is the hallmark of embryonic development. A better understanding of this interplay would enhance our ability to employ the principles of cellular self-assembly in the biofabrication of living constructs. In this section, we review characteristic morphogenetic mechanisms that are utilized in the biofabrication technology to be discussed in the next section.

3.1. Cell sorting

Cell sorting is a self-assembly process providing a common mechanism to establish cellular compartments and boundaries between distinct tissues [84–87]. To understand the molecular basis of cell sorting, let us consider lumen or cavity formation. As cells in an initially homogeneous cell population differentiate, they may become polarized and express cell adhesion molecules only on restricted parts of their surface. As a consequence, if minimization of configurational energy is the driving force in cell rearrangement, a lumen is bound to appear (figure 3).

Lumen formation is a simple manifestation of the differential adhesion hypothesis (DAH; [89, 90]), which postulates that cells of different type adhere to each other with different strengths (such as the polarized and non-polarized cells in figure 3). The DAH may also provide a simple molecular explanation for cell sorting: an initially mixed population of differentially adhesive cells, either due to quantitative ([90]; figure 4) or qualitative ([91, 92]; figure 5; see also figure 8) differences in cell surface adhesion molecules, evolves to a compartmentalized state in which the more adhesive cells aggregate and become surrounded by the less adhesive ones. As sorting requires the cells to be motile, this morphogenetic mechanism is most active during embryonic development, where adhesive contacts (through cell adhesion molecules such as cadherins or selectins) are not yet at the mature stage as in an adult organism (e.g. tight junctions, gap junctions) [88].

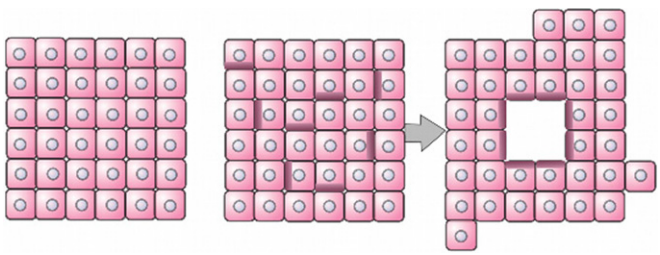

Figure 3. Schematics of lumen formation as a consequence of differential adhesion. The shading in some cells in the middle panel represents the lack of adhesion molecules on corresponding regions of the cells. These cells sort through the mixture and orient themselves to form a lumen or cavity [88]. (Figure reproduced with permission of Cambridge University Press.)

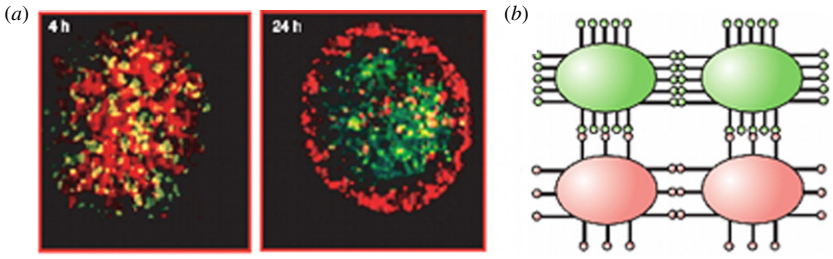

Figure 4. Sorting in a mixture of cells based on differential adhesiveness. (*a*) Sorting of two genetically transformed Chinese Hamster ovary (CHO) cell populations with ∼30% difference in *N*-cadherin expression (green cells contain more *N*-cadherins). Aggregates (200 μm in diameter) contain equal number (∼6000) of the two cell types (i.e. green and red), and sort within ∼24 h. (*b*) Schematic illustration of adhesion molecules (bars ending in circles) on the surfaces of the two CHO cell populations. To minimize the configurational energy, highly adhesive cells tend to group together. (Figure reproduced with permission of Cambridge University Press from [88].)

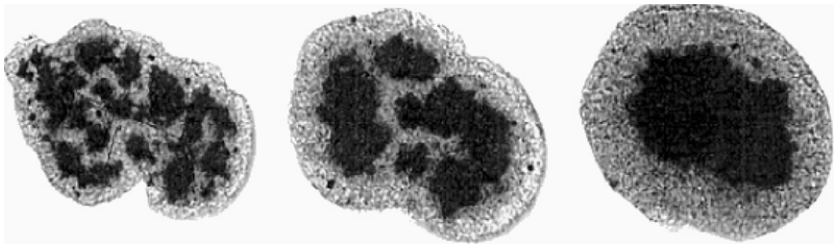

Figure 5. The images show the full cross-section of a 200 μm diameter spheroidal aggregate consisting of a mixture of chicken embryonic pigmented epithelial (dark) and neural retinal (light) cells. The three panels correspond to the times of 17, 42 and 73 h after the initiation of sorting [96]. (Figure reproduced with permission of *Proc. Natl Acad. Sci. USA*.)

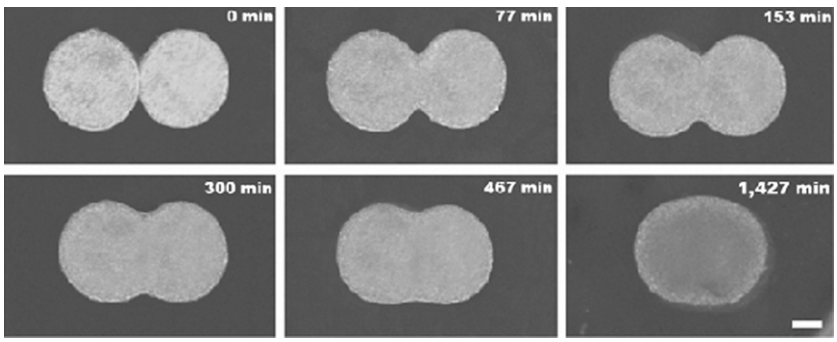

Figure 6. *In vitro* fusion of two embryonic cell aggregates. Snapshots of two apposed embryonic cushion tissue spheroids cultured in a hanging drop configuration fuse into a single spheroid over a period of ∼1 day [42]. Scale bar: 100 μm.

Though interpretation of sorting by DAH is appealing and elegant, recent developments suggest the situation may be more complicated; compartment boundaries between tissues may result from the synergistic effect of differential cell adhesion and cellular tensile forces generated by acto-myosin-dependent cell cortex tensions [91–95].

3.2. Tissue fusion

Tissue fusion is a self-assembly process in which two or more distinct cell populations make contact and coalesce. The fusion process underlies the formation of numerous structures in the embryo (for specific examples see [86]). Figure 6 illustrates the *in vitro* fusion of two multicellular aggregates [42]. A striking *in vivo* example is shown in figure 7 [81].

3.3. Apparent tissue liquidity

During sorting and fusion, the cellular system evolves in time from an initial state to a structurally and energetically more stable final state. It is an intriguing question what drives the underlying equilibration processes. As sorting and fusion strikingly resemble, respectively, the phase separation and coalescence in liquids, it was proposed that adhesive and motile cell populations have apparent liquid-like properties [98–100]. Based on this analogy, cell sorting and tissue fusion should be driven by apparent surface and interfacial tension. An apparent tissue surface tension has been measured using methods applicable to liquids [101–103] for a large variety of naturally occurring tissues [102, 104–106] and tissue cell aggregates [101, 103, 107–110].

It was suggested that the notion of apparent tissue surface tension is intimately related to DAH [108]: differences in

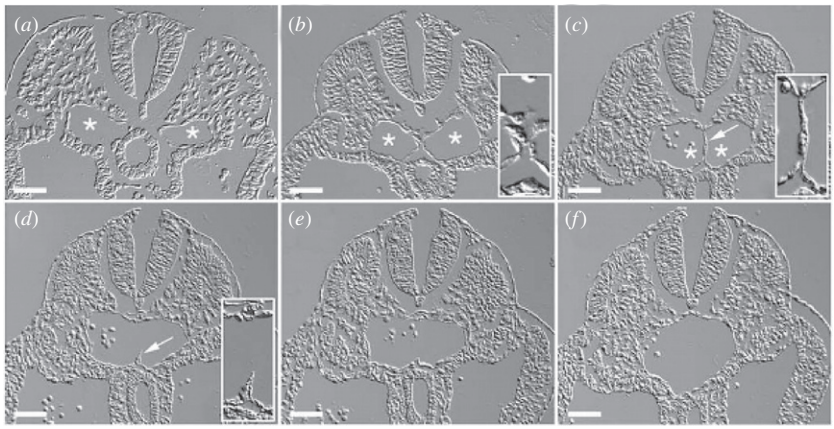

Figure 7. Formation of the descending aorta by fusion of the pair of dorsal aortae (81). Panels represent a series of cross-sections of the posterior–anterior axis in an E9.5 mouse embryo, arranged in a posterior to anterior direction. (*a*)–(*c*) Asterisks indicate the lumens of the paired dorsal aortae; arrow in (*c*) shows the cellular septum separating the two aortae. Insets in (*b*) and (*c*) show high-magnification views of the interface region between fusing dorsal aortae. (*d*) Arrow shows a remnant of the cellular septum. Inset in (*d*) shows a high-magnification view of the remnant of the cellular septum in the dorsal aorta. Scale bars: 50 μm. (Reproduced with permission of Wiley Interscience Publishing Co.)

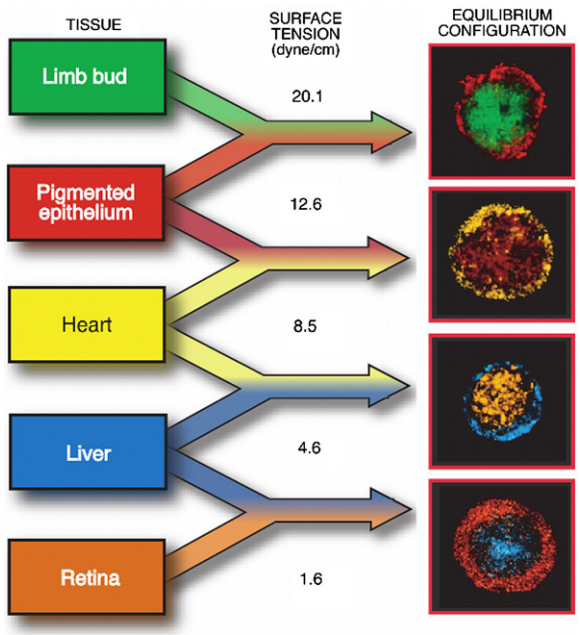

Figure 8. Correspondence between the sorted patterns and apparent tissue surface tension. Data are shown for five chicken embryonic tissues [88, 101]. (Figure reproduced with permission of Cambridge University Press from [88].)

apparent tissue surface tension are quantitative measures of differential cell adhesion. In particular, the measured values of these tensions are consistent with the observed sorting behavior: the tissue of higher surface tension is surrounded by the one of lower surface tension as shown in figure 8, similar to immiscible liquids, such as oil and water. Furthermore, using engineered cell lines it was demonstrated that apparent tissue surface tension is proportional to the number of cell surface adhesion molecules [108], implying that the more cohesive the tissue the higher its surface tension. The final sorted and fused tissue configurations, respectively shown in figures 8 and 6 can then be understood as equilibrium states with the lowest interfacial energy. As gravitational forces are negligible and other external forces do not act, in these states the cellular assemblies assume spherical shape.

The spontaneous rounding up of embryonic tissue fragments (figure 9) and multicellular aggregates was one of the first indications of the liquid-like nature of such cellular assemblies. Cellular spheroids are extensively used as close-to-physiological three-dimensional cell culture models that can be easily and reproducibly fabricated using a variety of rapid prototyping methods [45, 46, 111] (see section 4).

It is important to note that the concept of tissue liquidity establishes analogy as opposed to identity between true liquids and cellular assemblies: the motion of liquid molecules is driven by thermal fluctuations with characteristic energy k_BT (k_B is the Boltzmann constant and T is the absolute temperature), whereas the motion of cells is driven by metabolic energy resulting from ATP hydrolysis. Therefore, it is even more striking that not only the final sorted and fused configurations are liquid like, but so is the approach to these final states. Indeed, the time sequence shown in figure 6 can be described by the theory of highly viscous liquids [112–115]. Tissue liquidity, albeit a useful and convenient concept, is still not a universal morphogenetic principle. Processes that are consistent with tissue liquidity may act at one stage of early development but not at another. Indeed, apparent surface tension is typically a meaningful quantity only for a period of time that depends on tissue type [101]. Nevertheless, it is exciting that cellular properties can be summarized using just a few parameters (e.g. surface tension, viscosity, elastic constants) to describe large-scale tissue behavior with relevance to the fabrication of multicellular living constructs.

4. Engineering and fabricating tissues by bioprinting

Recently, several research groups have embarked on engineering three-dimensional living structures using bioprinting. Two main distinct technologies have emerged. One relies on the use of inkjet printing [116–124]. In this

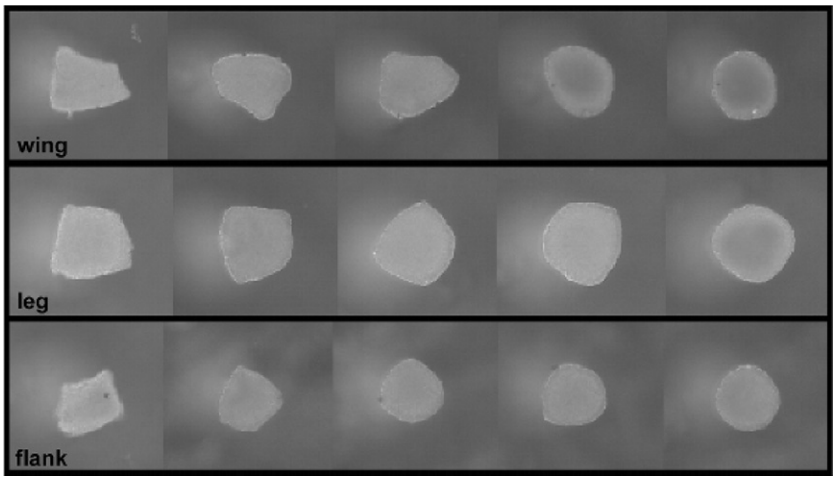

Figure 9. The spontaneous rounding of tissue fragments. The rounding of tissue fragments (~300 μm in size) of 4 day embryonic chick leg, wing and flank tissue that occurs within 24 h *in vitro*, is viewed as an indication of liquid-like behavior. The fragments were photographed successively after explant, and at 5 h, 11.5 h, 17.5 h and 24 h thereafter [104]. (Figure reproduced with permission of Elsevier, Inc.)

technology, either individual cells or small clusters are printed. The method is rapid, versatile and cheap. Its disadvantage is that it is difficult to assure high cell density needed for the fabrication of solid organ structures. Furthermore, due to the high speed of cell deposition, considerable damage is caused to cells, although the latest developments in the field have led to considerable improvement in cell survival. Finally, to achieve appropriate structural organization and functionality remain a challenge.

In the other approach, mechanical extruders [125, 126] are used to place 'bio-ink' particles, multicellular aggregates of definite composition into a supporting environment, the 'bio-paper', according to computer-generated templates consistent with the topology of the desired biological structure [42, 45, 97]. Organoids form by the postprinting fusion of the bio-ink particles and the sorting of cells within the bio-ink particles. The advantage of this technology is that the bio-ink particles represent small three-dimensional tissue fragments. Thus, cells in them are in a more physiologically relevant arrangement, with adhesive contacts with their neighbors, which may assure the transmission of vital molecular signals. The method employs early developmental mechanisms, such as tissue fusion and cell sorting. The disadvantage of the method is associated with the relatively high cost of the printers. Both inkjet and extruder bioprinting are compatible with rapid prototyping.

In this section, we review the latest developments in extruder-based bioprinting technology developed in our laboratory (figure 10). In particular, we first describe how the multicellular bio-ink particles are prepared and subsequently discuss how the special-purpose extruder printers deliver them according to computer-generated templates. For specificity we will use the process of engineering vascular grafts.

4.1. Fabrication of self-assembling, multicellular building blocks

Multicellular bio-ink building blocks are prepared from cell suspensions. They can be homogeneous, containing a single cell type or heterogeneous, made from a mixture of several cell types. Bio-ink particles typically used in our laboratory are either spherical or cylindrical in shape. Multiple methods have been described to prepare spherical aggregates [45, 46, 111, 127].

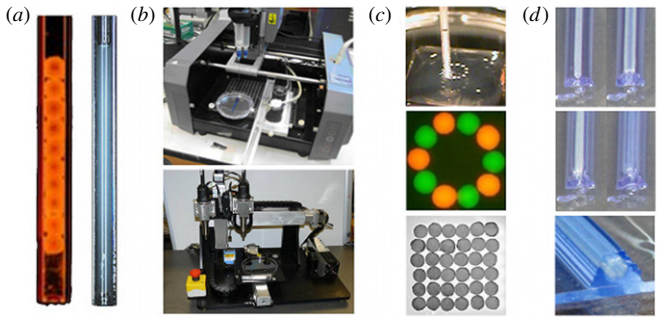

Figure 10. Components of the print-based tissue-engineering technology. (*a*) The bio-ink-filled micropipette printer cartridge filled with multicellular building blocks that can be spheroidal (left) or cylindrical (right) depending on the method of preparation. (*b*) The bio-printer. Three-dimensional printing is achieved by displacement of the three-axis positioning system (stage in *y* and printing heads along *x* and *z* (top: Neatco, Carlisle, Canada; bottom: Organovo-Invetech, San Diego)). (*c*) Spheroids are delivered one by one into the hydrogel bio-paper (itself printed) according to a computer script. (*d*) Layer-by-layer deposition of cylindrical units of bio-paper (shown in blue) and multicellular cylindrical building blocks. The outcome of printing (spheroids in panel (*c*), multicellular cylinders in panel (*d*)) is a set of discrete units, which postprinting fuse to form a continuous structure.

Here we briefly describe one method for the preparation of the spherical or cylindrical units (figure 11). The cell suspension is centrifuged and the resulting pellet is transferred into a capillary micropipette. After a short incubation in medium at 37 °C, cell–cell interactions are restored and the cylindrical slurry becomes sturdy enough to be extruded into liquid. The spherical building blocks are obtained by mechanically cutting uniform fragments that spontaneously round up as a manifestation of tissue liquidity. If the slurry

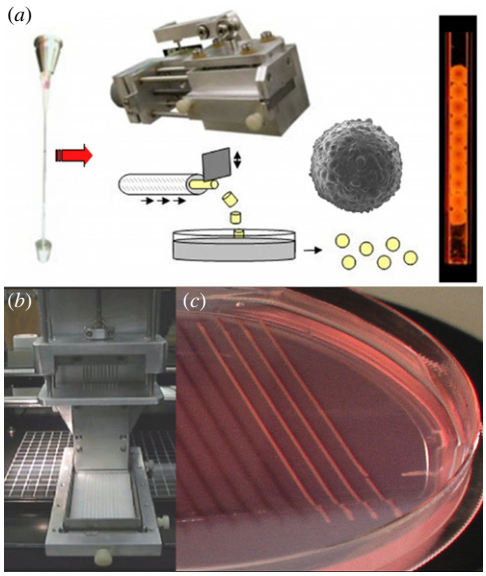

Figure 11. Preparation of the multicellular building blocks. (*a*) Preparation of spherical building blocks. The cell slurry extruded from a micropipette is cut into cylindrical fragments (with identical diameter and height) of equal size using a custom device. Spherical bio-ink particles are formed by rounding of the cylinders upon overnight incubation on a gyratory shaker. A scanning electron microscopy picture of a 500 μm spheroid composed of endothelial cells is shown. The spheroids are packaged into the bio-ink-filled micropipette printer cartridge just before printing. (*b*) Preparation of cylindrical building blocks. Up to ten multicellular slurries can be simultaneously extruded into a non-adhesive agarose mold using a customized attachment to the bioprinter. After overnight maturation, the multicellular cylinders are strong enough to be printed.

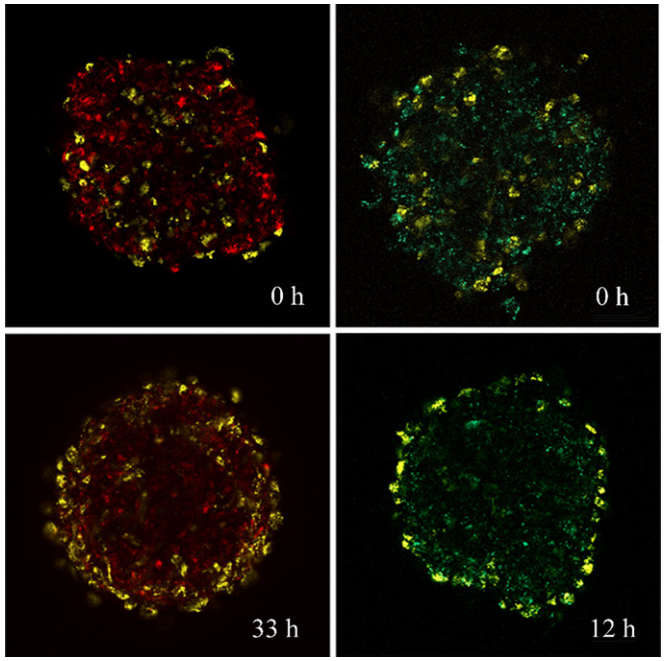

Figure 12. Time evolution of cell sorting in multicellular aggregates. Aggregates composed of endothelial cells (yellow) and fibroblasts (red) (left column) and endothelial cells (EC) (yellow) and smooth muscle cells (SMC) (green) (right column) are shown. The sorting pattern can be attributed to the differences in the apparent surface tension of multicellular assemblies prepared from the two cell types, similarly to the situation shown in figure 8 (endothelial cell aggregates: 12 dyne cm^{-1}; fibroblast aggregates: 72.7 dyne cm^{-1}, smooth muscle cell aggregates: 279 dyne cm^{-1}).

is composed of multiple cell types, sorting and rounding will occur in parallel. As for embryonic tissues, the sorting behavior is driven by differences in tissue surface tension of the cell aggregates (figure 12). The fabrication of cylindrical building blocks requires maturation of the slurries in a non-adhesive mold overnight to improve their cohesivity. Automation of the deposition step into the mold has been achieved and was important for the high quality of cellular cylinders and the rate of their production.

4.2. Bio-ink deposition

The extrusion-based bioprinting described here represents an automated deposition method that enables the building of 3D custom-shaped tissue and organ modules without the use of any scaffold. In this way, a fully biological construct is generated that is structurally and functionally close to a native tissue. Spherical or cylindrical multicellular units—the bio-ink—are delivered according to a computer-generated template together with hydrogel—the bio-paper—serving as support material.

Different deposition schemes have been employed for 3D tissue bioprinting as the technology evolved. The scheme shown in figure 13 was used initially to establish proof of concept. Despite some successful outcomes [42], the rapidity, reproducibility and scalability of the technique were challenged by a few shortcomings.

One limitation of this early system was that the success of printing depended strongly on the control of the gelation state of the collagen-hydrogel layers. Uneven gelation compromised the spatial accuracy of the construct in particular for constructs thicker than a few layers [42]. In addition, collagen was remodeled by the cells and integrated into the final structure, such that its removal was challenging. Another limitation arose during printing constructs of larger size and more complex pattern (i.e. branching tubes). The preparation of spheroids in large quantities (>1000 for branched tubular structures) became excessively time consuming. Finally, the manual filling of the micropipette printer cartridge with the cellular spheroids (one by one) represented a serious challenge (e.g. it had to be assured that no gaps between the spheroids appeared upon their aspiration into the micropipette).

To overcome the above limitations, we replaced the collagen sheets with agarose rods (figure 14) and we used cylindrical multicellular building blocks instead of spheroids (figures 10 and 14). The agarose rods are formed *in situ* and deposited by the bioprinter automatically, rapidly and accurately. Agarose is an inert and biocompatible hydrogel that cells neither invade nor rearrange. The agarose rods kept their integrity during post-printing fusion, and were easily removed to free the fused multicellular construct.

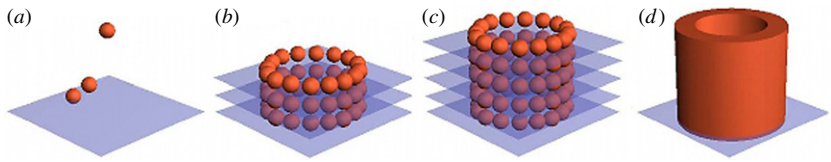

Figure 13. Layer–by-layer deposition. (*a*) A sheet of biocompatible hydrogel is printed, and the building blocks are embedded into the hydrogel. (*b*) and (*c*) The alternate deposition of layers of hydrogel and building blocks is pursued according to the predefined blueprint of the desired 3D structure (here, a tubular construct). (*d*) Fusion of the building blocks and removal of the hydrogel result in a hollow tube after a few days.

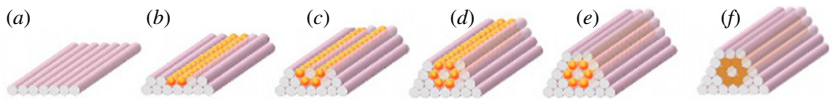

Figure 14. Horizontal layer-by-layer deposition of the building blocks. (*a*)–(*e*) A possible deposition scheme for a tubular structure built of agarose rods and spherical multicellular building blocks. (*f*) The same tubular configuration printed with cylindrical multicellular building blocks.

4.3. Self-assembly of the building blocks

A living cell-based construct that results from the described printing process and the postprinting fusion of the bio-ink particles is subsequently placed in an incubator where it achieves its final 3D structure and through maturation develops appropriate biomechanical properties. Proof of concept studies have been carried out to build tissue toroids, thick sheets and straight and branched tubes ([42, 45]; figure 15).

It was demonstrated that no biological functionality was lost in the bioprinting process when the sheet obtained by the fusion of chick cardiac cell spheroids exhibited synchronous macroscopic beating throughout the construct. When endothelial cells were mixed with the cardiac cells, self-assembly led to the formation of vessel-like conduits [42].

Our current efforts are focused on the bioprinting of small and intermediate diameter blood vessel substitutes ([45], figure 16(*a*)). As the print-based technology exploits the intrinsic self-organizing properties of cells and tissues, it incorporates some of the natural vessel forming processes. During embryonic vasculogenesis and angiogenesis, the initial steps are performed by the ECs as they form the elementary tubular conduits. Under shear stress, the ECs secrete the growth factors PDGF and TGFβ and the associated molecular signals are responsible for the recruitment of SMCs and the induction of ECM deposition (i.e. collagen and elastin). In particular, TGFβ plays a key role in the production of elastin, an ECM component that not only is responsible for the elastic properties of the vessels but also regulates the sequestration and activation of the growth factors [128].

Using the described print-based technology, tubular structures can be built from mixtures of randomly distributed ECs and SMCs, which offers a unique opportunity to exploit the EC/SMC interaction from the initial step of vessel formation. Endothelium is expected to form during the postprinting fusion and sorting (figure 16(*b*)). Applying flow to the forming endothelium in the early stage will stimulate ECs to behave as they do *in vivo*. The anticipated benefits will be the production of a more physiological ECM (both in composition and organization), better attachment of the endothelium and faster maturation. To mimic the blood vessel structure, a double-layered vascular tube has been constructed (figure 16(*c*)).

In conclusion, the novel print-based tissue-engineering technology has several distinct features with great potential for the generation of tissue and organ structures: (i) it represents an approach for producing fully biological (scaffold-free) small diameter vessels; (ii) it utilizes natural shape-forming (i.e. morphogenetic) processes, that are present during normal development; (iii) it can provide organoids of complex topology (i.e. branching tubes) and (iv) it is scalable and compatible with methods of rapid prototyping.

5. Future perspectives

While regenerative medicine is far from providing 'replacement parts' and tissue regeneration therapies ready to be used in patients, we seem to be getting increasingly closer to the development of treatment modalities for re-establishing tissue structure and function. The rapid development of the field is largely driven by the medical needs of our aging population, and by recent developments in stem cell biology and technologies of cell and tissue engineering. In the last few years, tissue engineering went through some major changes that might be indicative of the future trends in this field.

Overall, a largely empirical approach to tissue engineering ('tissue try this', as cleverly described by Shannon Dahl) is being replaced by the development of developmentally inspired technologies (approaches based on bioprinting and self-assembly being remarkable examples). Consequently, one major focus is on the cells. With the living cell being the ultimate 'tissue engineer', our effort is being directed to providing the conditions, so that the cells do the engineering. Another focus is on technology. After major breakthroughs in stem cell biology, we are now developing technologies for 'instructing' the cells to regenerate defective tissues by providing a highly engineered environment.

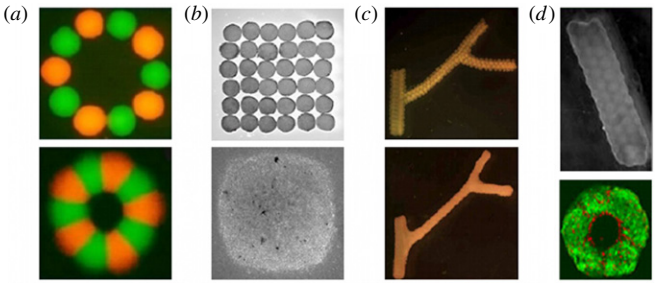

Figure 15. Printing of geometrically complex multicellular constructs. (*a*) 500 μm bio-ink particles (top), composed of Chinese Hamster ovary (CHO) cells were printed on 1.3 mg/ml collagen bio-paper, in circle (aggregates are labeled alternatively with red and green dyes). A fused toroid forms after ∼60 h (bottom) [42]. (*b*) Chick cardiac cell aggregates printed into sheet (top). A fused sheet, after focus ∼70 h (bottom) [42]. (*c*) A branching conduit printed using spherical units; bottom panel shows the fused structure. (*d*) A 12-layered printed CHO tube using spherical units (top). Cross-section of the fused tube made with units composed of SMCs (green) and ECs (red) (bottom).

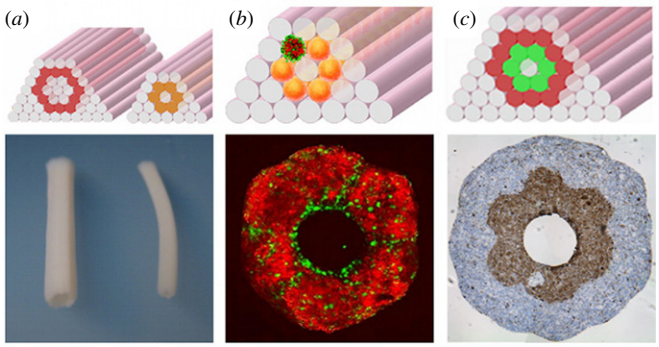

Figure 16. (*a*) Design templates (top) and fused constructs (bottom) of different vessel diameters built with cylindrical bio-ink. (*b*) The top image shows a template to build a construct with spheroids composed of SMC (red) and ECs (green). A transversal section after fusion (bottom) shows that the lumen is composed predominantly of endothelial cells. (*c*) Template to construct a double-layered vascular tube (top). The inner layer is constructed of SMC building blocks (green), the outer of fibroblast building blocks (red). The transversal section (bottom) shows fusion and the segregation of the two cell types mimicking the media and adventitia of blood vessels.

These focus points are illustrations of the paradigm shift in the field toward the establishment of a 'biomimetic' approach, that is based on the premise that unlocking the full biological potential of stem cells (*in vitro* or *in vivo*) necessitates an environment similar to that present during native development. This paradigm is guiding many current tissue-engineering approaches, including those based on cell self-assembly that holds promise of building fully biological tissues with structural and functional specification. We described the potential of the self-assembly-based technology for the formation of a hierarchically defined vascular tree perfused with blood, one of the main unsolved problems in tissue engineering. It is highly possible that in the future this technology can also help build other complex tissues, such as stratified and anisotropic structures consisting of multiple cell types and tissue subunits. For this to happen, we will need to continue to cross the boundaries between the disciplines, and take advantage of the synergism of developmental and adult biology, biomaterials science, biomedical engineering and medicine.

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