Scaffold-Free Biofabrication

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Abstract

Tissue engineering and regenerative medicine have met great scientific, medical, and technological advances in the past decade. Most methods combine scaffolds, such as polymers, and living cells to make implantable structures that will integrate and heal the host's tissues. More recently, alternative scaffold-free approaches have started to emerge. This chapter provides an overview of the current scaffold-free systems, advantages, challenges, methods, and applications. Scaffold-free tissue artificially produced in the lab using patients' own cells has already been successfully used in heart and blood vessel regeneration at a small scale. New techniques and approaches are being developed, not only in terms of assembling cells and structures but also in terms of new equipment, namely for 3D bioprinting. Both primary and stem or iPSC-derived cells are used to assemble artificial tissues that are currently being tested in vivo and in vitro. These engineered constructs have numerous applications, such as regenerative medicine, disease models, and drug testing.

1 Introduction

Until recently, most studies on tissue function, whether normal or in pathological conditions, were performed using either in vivo models that do not always behave in the same way as the human body does and can at times raise ethical issues (Festing and Wilkinson 2007; Mak et al. 2014; Denayer et al. 2014) or 2D in vitro models, which fail to provide the adequate environment for cells to maintain their normal features and behavior. A step forward was taken when 3D cultures were developed where cells are cultured with or without exogenous extracellular matrix, in a much more in vivo-like environment (Edmondson et al. 2014).

In recent years, the fields of tissue engineering and tissue regeneration have undergone rapid growth and evolution to overcome these difficulties and create more realistic models and solutions. Many materials have been developed as scaffolds, which provide a template, with predefined geometry, where cells can attach, expand, and even differentiate (O'Brien 2011). Alternative approaches, termed scaffold-free, are also employed in regenerative medicine and tissue engineering.

2 Scaffold-Free Tissue Engineering

Scaffold-free methods had their infancy in the early 1990s (Yamada et al. 1990) but have somewhat developed slower than those using scaffolds, mainly due to the structural challenges of building complex geometries and relatively big structures without rigid support (Czajka et al. 2014). The advent of new technologies and better understanding of developmental processes and cell manipulation have led to advances and expansion of these methodologies.

The definition of scaffold-free tissue engineering still remains unclear and controversial, loosely meaning the production of living tissue using cells only and relying on them to produce their own matrix and architecture. Athanasiou et al. (2013) have stated that "scaffoldless tissue engineering refers to any platform that does not require cell seeding or adherence within an exogenous, three-dimensional material."

In a review concerning current scaffold-based and scaffold-free bioprinting methods, decellularized matrix components, as well as hydrogels and microcarriers, were classed as scaffold-based bioprinting. As for the scaffoldless counterpart, it was pointed out that the absence of hydrogels and higher cell density, more similar to naturally occurring tissues, affords quicker fusion and maturation (Ozbolat 2015). Elsewhere, it is stated that the current two methods for making scaffold-free tissue engineered vascular grafts are sheet seeding and decellularizing/recellularizing (Lee et al. 2016). The latter was considered scaffold-free, as the scaffold used to create the vessel was highly degradable, leaving behind a robust tissue that could be decellularized and applied in vivo, with or without recellularization. Complete organ decellularization has also been achieved for kidney, liver, lung, and heart (Orlando et al. 2013; Gilpin et al. 2014; Guyette et al. 2014; Mazza et al. 2015). If the first example, after decellularization, is considered a scaffold-free approach, by comparison, so should the latter ones. However, using a scaffold, even if of natural origin, to produce a scaffold-free structure seems rather contradictory. Additionally, hepatocyte spheroids aggregated without the addition of exogenous scaffolds and then encapsulated in hydrogels for further assemblage and implantation (without a bioprinting step) were also classed as scaffold-free. The distinction used in this chapter, whether referring to the initial process of cell aggregation or subsequent steps, such as bioprinting, accepts that the final tissue should remain scaffold-free at the time of implantation or testing. Temporary scaffold or hydrogel materials may be used, so long as they do not form part of the final tissue. They only lend structural support to keep the structure together until the cells or spheroids start to grow and fuse. Ideally, these substances should remain in the living structures for as little as possible and be easily removable by either physical peel or chemical or thermal processes.

Another concept, or area of research, that relates to tissue engineering and regenerative medicine is biofabrication. With the recent explosion in the development and availability of 3D bioprinters and associated methods, materials, equipment, and accessories, "biofabrication" has expanded accordingly. Presently, and in this context, biofabrication has been widely used as synonymous of 3D bioprinting and terminology is somewhat confusing. However, the concept is not new and represents different processes and materials in different disciplines. A definition harmonizing bioprinting and bioassembly for tissue engineering and regenerative medicine has been recently proposed as "the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through Bioprinting or Bioassembly and subsequent tissue maturation processes" (Groll et al. 2016). Thus, the two concepts are complementary.

In the same way, more traditional definitions of tissue engineering, such as "understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use" (MacArthur and Oreffo 2005), are now being expanded to other applications, such as in vitro models for disease or drug screening (Vunjak et al. 2014). As the tissues made for clinical uses are becoming more realistic, it makes sense that they can also be used as in vitro models. Therefore, in a wider interpretation of these notions, scaffold-free stand-alone functional models, such as organoids, that do not necessarily constitute the building blocks for bigger structures or are produced by automated methods will also be discussed in this chapter.

3 Classification of Existing Scaffold-Free Systems

Existing scaffold-free systems can be classified according to the type of building blocks used (cell sheets, isolated single cells or spheroid cell aggregates) or the processes involved in the formation of the artificial tissues or building blocks.

Self-organization is achieved by using external forces (for example, bioprinting and cell sheets), and self-assembly relies on spontaneous events without any external forces (nonadherent substrates are used so that the cells can carry out all the events with minimal intervention, such as spheroid formation). Both processes of self-organization and self-assembly produce highly biomimetic tissues that are capable of looking and behaving in a similar fashion to the native tissues they recreate, thus holding potential for clinical applications (Athanasiou et al. 2013).

The very first cell sheets for clinical application were developed by Rheinwald and Green in 1975. They used keratinocytes to produce sheets that were applied onto sites of severe burns. Over the years, these grafts have been refined and are now in clinical use, approved by the FDA (Phillips 1998).

Fifteen years later, an enzyme-free method for cell sheet detachment was developed, using poly(*N*-isopropylacrylamide), which is temperature-responsive (Fig. 1a). Below 32 °C the material becomes hydrophilic, which results in cell detachment (Yamada et al. 1990; Okano et al. 1993). The method can be used for more fragile sheets and does not require feeder cells. This has successfully been used to produce implantable engineered tissues for clinical repair, such as skin (O'Connor et al. 1981), cornea (Nishida et al. 2004), esophagus (Ohki et al. 2009), and heart muscle (Sawa et al. 2012). Recently, the same group has developed an automated cell culture system and fabricated corneal epithelial cell sheets were successfully implanted into rabbits, allowing regeneration on a limbal epithelial stem cell deficiency model (Kobayashi et al. 2013).

Cell sheets can be produced individually and then rolled on top of each other (Fig. 1b). Initial studies have shown that it was possible to build blood vessel-like structures without the use of scaffolds. Overlaying a sheet of fibroblasts over a sheet of smooth muscle cells and then seeding endothelial cells on the lumen produced a three-layered structure, able of synthesizing extracellular matrix proteins usually found in blood vessels, such as elastin and collagen. Furthermore, the cells expressed the correct cell differentiation markers and were able to perform functions such as platelet adhesion inhibition (L'Heureux et al. 1998). The most notable advancement

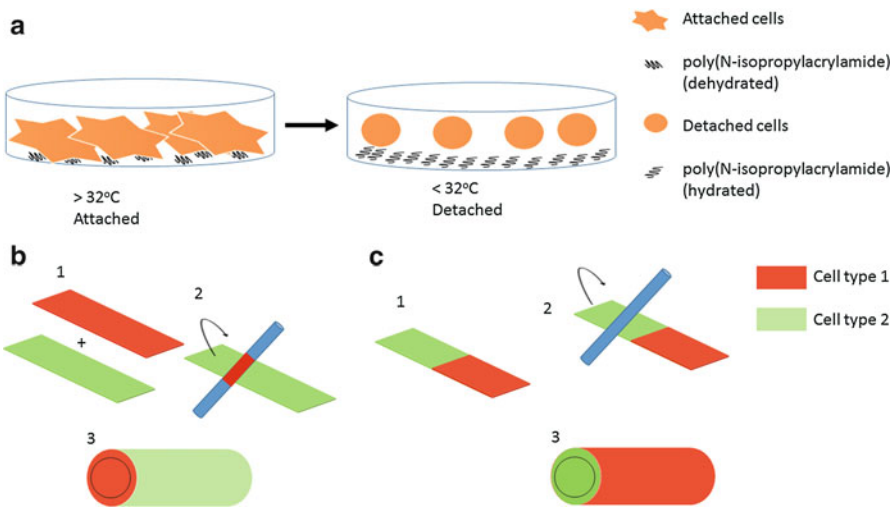

Fig. 1 Scaffold-free systems: cell sheets. (**a**) Poly(*N*-isopropylacrylamide) is a temperature-responsive polymer that is used to coat cell culture dishes. In aqueous solutions, above 32 °C, the molecule is dehydrated, which allows cell attachment. Below that lower critical solution temperature, the molecule will be hydrated and swollen, which results in cell detachment. (**b**) Cell sheets of the same or different cell types can be detached from the culture vessel and rolled on top of each other to form multilayered tissues. (**c**) Alternatively, two or more cell types can be grown in different areas of the same sheet, and then rolled onto each other in a single-step procedure

was the strength of the construct, which could cope with physiological pressure and allowed in vivo grafting. This was where promising earlier attempts had failed (Weinberg and Bell 1986; L'Heureux et al. 1993; Hirai and Matsuda 1996). When this approach was first developed, it would take around 5 months to obtain the final product. This technique is still widely used and has been refined along the years.

An alternative single-step method has also been developed, where two cell types, vascular smooth muscle cells and fibroblasts in this case, were separately seeded on each half of a gelatin-coated plate, divided by a spacer, which was removed 24 h later. The cells were allowed to grow and migrate towards each other, to form a continuous monolayer with two distinct halves. When ready, the sheet was rolled onto a tubular support, allowing the fibroblasts to be placed on top of the smooth muscle cells, without the need for extra steps (Fig. 1c). Compared to the traditional two-step method, apart from saving time, these constructs showed improved mechanical properties, such as strength and viscoelastic behavior (Gauvin et al. 2010).

4 Aggregation/Spheroid-Based Approaches

Self-assembly approaches such as spheroid formation can use one (homocellular) or multiple (heterocellular) cell types to produce spheroids of controllable size and shape. The aggregates can form by a variety of methods, such as the rotating wall

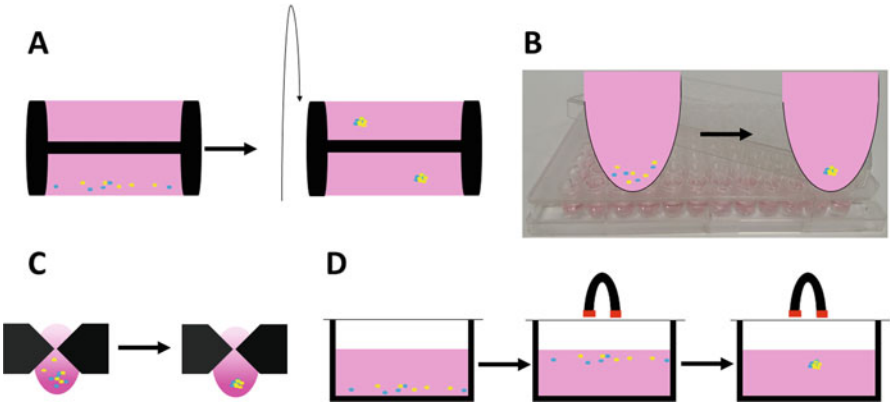

Fig. 2 Scaffold-free systems: spheroids. Homo- or heterocellular spheroids can be obtained by a variety of methods. (**a**) The rotating wall vessel consists of a cylinder containing culture medium and cells, with a gas-permeable membrane at the center and revolves around a horizontal axis. This allows the cells to experience a free-fall environment and form aggregates, with or without microcarriers. (**b**) Ultralow attachment wells are coated with hydrophilic molecules that do not allow cell attachment to the bottom or the walls. Due to this inability and gravity, seeded individual cells will converge at the bottom and form spheroids. (**c**) The hanging drop method consists of placing drops of cell suspension at the bottom of a tissue culture dish lid or a specialized multiwell plate so that they are suspended. Then, the individual cells will cluster to form aggregates. (**d**) Cells can also be preincubated with magnetic particles and levitate in the presence of a magnet. They will subsequently spontaneously aggregate and form spheroids

vessel, micromolding, ultralow attachment plates, pellet, hanging drop, and magnetic levitation.

The rotating wall vessel bioreactor was devised by NASA to produce cartilage in a microgravity environment, which is simulated by the rotation of a circular vessel around a horizontal axis (Freed et al. 1997). Isolated individual cells added to the system, mixed with porous beads or without beads, have access to fluid shear, oxygen and nutrients and slowly start to form 3D structures. These have been made for a selection of tissues and used for a wide variety of studies, such as small intestine, bladder, lung, and liver (Barrila et al. 2010) (Fig. 2a).

Some cells, when confluent, have the capacity to produce high amounts of extracellular matrix. This property has been explored to create scaffold-free, matrix self-producing 3D structures made up from chondrocytes, treated with transforming growth factor-beta and other growth factors and matured into shape in molds. Proteoglycan and collagen were identified and the overall strength allowed implantation into a pig articular cartilage defect model, which was well integrated (Miyazaki et al. 2010).

Cells seeded onto U-shaped polystyrene plates, which do not allow cell attachment, will deposit onto the bottom and form spheroids (Kelm et al. 2010) (Fig. 2b). Depending on the combination of cell types, the resulting architectures may be more or less organized (Mironov et al. 2009). A variation of this method includes spinning the cells down, forcing them to form spheroids (Baraniak and McDevitt 2012).

Another way of obtaining spheroids consists of adding PBS to a petri dish, to generate a wet chamber and pipetting drops of cell suspension onto the inside of the lid, hanging upside down. Due to the effect of gravity, the cells will spontaneously aggregate (Foty 2011; Jorgensen et al. 2014). An automated version of this method, using a 384-well plate with upper and lower reservoirs for PBS and the hanging drops, has been developed (Tung et al. 2011) (Fig. 2c).

Super paramagnetic iron oxide nanoparticles can be incubated with cells so that they get internalized. After that, the cells are harvested and transferred into ultralow attachment plates. Then, aggregates can immediately start forming by holding a magnet either on top or under the plates for a few hours (Tseng et al. 2015; Leonard and Godin 2016) (Fig. 2d).

Complex scaffoldless structures may in some cases be more challenging to achieve than if scaffolding materials were to be used to provide support and shape. Therefore, the latter rely on the cells' ability to build their own 3D network. The final structure can be achieved by either placing the cells in molds until they aggregate and form a structure that will hold on its own, or small aggregates can be combined by bioprinting.

Spheroids obtained by any of the methods described above are suitable for subsequent bioprinting, into bigger and more complex structures. Depending on the method/equipment used, they need to be within a strict amplitude of values for size and shape, compatible with the software and nozzle. If they are to be printed, aggregates must be only a few days old, otherwise they will lose the ability to fuse. Bioprinting of scaffold-free cell materials can be expensive and time-consuming when making the spheroids, as a very high number of cells are required. However, these support faster and better tissue growth.

Spheroids themselves can also be used as models for tumors or even simplified organs or functional units of organs. Cells in 3D cultures exhibit higher expression of adhesion molecules (among others) and the native tumor microenvironment and matrix, as well as cell interactions, are more closely recreated than in their 2D counterparts. Spheroid tumor models are currently done with or without scaffolds. These spheroids can acquire very different shapes, varying from round to stellate and depend on the cell type/state used and culture conditions. Structurally, spheroids can resemble a tumor, with hypoxia, necrosis, and metabolite accumulation at the core region, a middle layer of quiescent cells, and an outer proliferating rim (Nath and Devi 2016).

5 Preparation of Multicellular Spheroids and Construct Design

Scaffold-free approaches largely rely on the cells' intrinsic ability to spontaneously remodel and differentiate into naturally occurring systems, as happens during embryonic development. The preparation of spheroids can be done by several methods, but they all rely on the fact that dissociated cells can regroup to form aggregates. This is a survival mechanism that prevents anoikis (apoptosis due to the lack of correct cell/matrix attachment). Depending on the cell type, culture

conditions and method chosen, aggregation normally takes between 24 and 72 h. The formation of a spheroid in an ultralow attachment well can be visualized on Video 1.

3D bioprinting has made depositing cells or cell aggregates easier, faster, more accurate, and reproducible than by hand. With the aid of software, users can design the structures they want to obtain and the equipment will then deposit the various materials at very precise positions. There are three main stages to consider when designing and fabricating 3D bioprinted tissue: preprocessing (digital design of the final construct), processing (the actual printing step), and postprocessing (maturation stage) (Mironov et al. 2003). Computer-aided design software can be used to freehand design simple structures, such as a tube composed of 20 rows of 32 spheroids each, or more complex designs based on 3D images acquired from patients, for example, CT scan, MRI, angiography, and echography (Sun and Lal 2002; Murphy and Atala 2014).

Printing can be achieved by using cells only or cells combined with a material that will confer initial structure but that can be quickly removed (thermo-reversible, dissolved chemically or physically pulled away). Flat tissues are easier to print than tubular or more intricate shapes. Vascularization, especially an internal network of microcapillaries required to keep the tissue alive and healthy, is still a challenge. Also, during this step, it is important to choose methods/equipment and materials that ensure cell viability. The design will have to take all these factors into account.

When printing scaffoldless structures, spheroid fusion, cell migration, and remodeling, as well as deposition of extracellular matrix and eventually cell differentiation, are expected to occur spontaneously. A vast body of literature currently describes how these processes occur during development and tissue engineering is now even considered a branch of applied developmental biology (Ingber et al. 2006). Embryonic tissue (or spheroids for bioprinting), composed of different cell types, can be compared to a viscoelastic fluid. When two immiscible liquids are mixed together, they will sort themselves apart. In the same way, different cell types mixed together in a spheroid will migrate until one surrounds the other, a process driven by surface tension and adhesive interactions (Beysens et al. 2000). When designing the final construct, building blocks can be made all the same or with different cell compositions, and deposited at distinct locations, which accelerates the sorting process. Depending on the printing equipment, spheroids may be picked up individually (Itoh et al. 2015), combined with agarose rods (Norotte et al. 2009), or mixed with a hydrogel.

Additionally, before printing, it is important to prepare the building blocks in advance (cells or spheroids) and any additional materials required. Depending on the resources available, construct size and intended application, different cell types and sources can be used (for example, iPSC, primary cells, cell lines, or adult stem cells).

Construct design will depend on the software available, printer resolution, type and size of printing material, application, cell origin and differentiation status, type of printer, bioink, building blocks, and assembly method. In vitro models, at least for some structures, will need finer detail than structures printed for transplantation, where the host can provide the right environment and colonizing cells (Itoh et al. 2015).

6 Bioprinting Methods and Equipment

Since the first mention of using a computer-assisted inkjet printer to deposit cells and proteins at precise positions (Klebe 1988), technology has flourished and recent years have seen an enormous progress in 3D bioprinting methods and equipment, both scaffold-based and scaffold-free. Printing living, viable cells, however, is more complex than printing nonliving materials, such as plastic or metal.

Bioprinters can be classified according to their use of laser, into laser-assisted or laser-free (inkjet, microextrusion). Variations of the latter are used for scaffold-free 3D bioprinting, most commonly mechanical microextrusion (Murphy and Atala 2014). Ideally, in scaffold-free printing methods, living cells are deposited directly onto a substrate, without the addition of other materials. Then, expansion, fusion, and migration processes occur to produce the final structure. In this case, the "bioinks" would be cell pellets, tissue spheroids, or tissue strands. Methods using these starting materials print high cell densities, which means cell fusion and remodeling, as well as extracellular matrix production, can start right after printing (Achilli et al. 2012).

A wider nozzle than those of inkjet printers allows for a larger number of cells to be printed at one time. With the addition of extra nozzles, different materials can be printed usually sequentially or in alternation. Cells or spheroids can be printed between layers of hydrogel, which confer structure and will then be removed after maturation, rendering the constructs scaffold-free. In other cases, cells can be printed together with hydrogels or oils.

The most widely used printers use extrusion, where cell spheroids are first loaded into a tubular reservoir/dispenser, ready for printing. With this method, variable amounts of oils or a hydrogel that does not allow cell adhesion usually need to be added. This is because cell culture medium would be too liquid to both deposit the cell materials in accurate positions or to keep them homogeneously distributed while on the cartridge. On the other hand, this technology is not compatible with printing cell masses on their own. The cell material to other materials ratio is still much higher than in bioprinting methods that are classed as scaffold-based. Another difference is that in scaffold-based printing the scaffold is there to be part of the structure and keep the cells in place. On the other hand, carrier liquids used in scaffold-free bioprinting are used to keep the building blocks from fusing prematurely, as well as an extrusion medium. They are not intended to be part of the final product (Marga et al. 2011).

After loading, cells and carrier are extruded by means of mechanical pressure. As they make their way out of the pipette, they are deposited onto a mold (which will not be part of the final construct), so that they can fuse, maturate, and acquire a predetermined architecture. This information will have been previously inserted into the program (usually CAD-based), so that the printer can deposit the cells at the correct locations. The same approach can be used to print less mature aggregates of cells and even cell strands (Ozbolat 2015).

Apart from the need of a carrier medium where the cells are delivered, loading the dispensers can be challenging. When loading all the spheroids into the pipette/

cartridge at once, time is of essence, as they may start to fuse while inside the dispenser, despite the low adhesion medium. Conversely, after printing, they need to be close enough to fuse to each other, or else gaps will be left.

Microcarriers will have degradation issues similar to those of hydrogels. Additionally, they need to be adhesive enough for fusion after printing and not too adhesive as not to block the nozzle.

A newer method, called the Kenzan method, does not rely on scaffolds, molds, or even carrier liquids to ensure correct spheroid positioning. Needle arrays (Kenzan) with different sizes and arrangements are placed in PBS and a nozzle will aspirate each spheroid individually and place it in the correct xyz coordinates, as determined by the design program. The stainless steel needles have a diameter of 100–200 μm and a pitch of 300–400 μm. Before and during printing, the bioprinter (Regenova) is able to check the spheroids for several parameters, such as size (diameter) and smoothness (roundness). This allows the machine to decide which spheroids are suitable for aspiration and which ones are not. Depending on the spheroid size, weight, and robustness, aspiration parameters may also be adjusted. The collected spheroids are skewered through the needles and when the process is completed the needle array is transferred into culture medium and incubated for initial maturation, after which the needles are removed. Therefore, this printing method does not use any chemicals that make up bioinks, nor does it put the cells through great temperature changes, which increase cell viability. Another advantage is that the spheroids are produced and printed individually, so they will not start fusing while being manipulated and loaded for printing.

7 Applications

Scaffold-free approaches are now being employed to generate artificial tissue representative of many different organs, resourcing to a variety of techniques. Below are a few examples of scaffoldless tissues that have been developed and are currently being used for both research and clinical applications.

7.1 Blood Vessels

Engineered blood vessels can be used as vascular grafts, models for disease and drug discovery or as part of vascularized structures for research or therapy.

Mesenchymal stem cells are an attractive source of material because they can differentiate into several cell types, such as endothelial cells, smooth muscle cells (Silva et al. 2005), osteocytes, and chondrocytes (Pittenger et al. 1999), among others. In 2012, tissue-engineered vascular grafts made from rabbit mesenchymal cell sheets were autologously implanted into the common carotid artery. After 4 weeks, the graft showed good patency and had successfully integrated the native vessel. Endothelialization was confirmed and the overall structure was similar to that of a real artery (Zhao et al. 2012). Since this discovery, many others have used these

cells. Jung et al. (2015), for example, have produced 1 mm inner diameter tubular structures using human mesenchymal cell sheets cultured on a rotating wall bioreactor. They later added human endothelial progenitor cells to form an endothelium, and further cultured them in a perfusion system. The constructs showed vasoconstriction and vasodilation, as well as nitric oxide release and adhesion of HL-60 to the lumen, a promyelocytic leukemia cell line.

A different strategy consisted of using spheroids made from primary human umbilical vein endothelial cells (40%), human aortic smooth muscle cells (10%), and normal human dermal fibroblasts (50%) to bioprint tubular tissues using the Kenzan method. These were matured in a perfusion system and implanted into the abdominal aortae of nude rats. After histological analyses, it was confirmed that both remodeling and lumen endothelialization had occurred (Itoh et al. 2015).

Human vascular grafts produced by the cell sheet method have been used in clinical trials as access for hemodialysis (McAllister et al. 2009). After a 6 month follow-up of the first 10 patients, patency remained 60% after initial 78% at 1 month postoperation. These results are encouraging; however, faster and cheaper methods need to be developed, as it took 6–9 months to produce these grafts.

7.2 Kidney

Kidneys are complex organs, composed of approximately 30 different cell types, required to create diverse structures, in order to perform functions such as pH regulation, electrolyte and fluid balance, hormone production, mineral absorption, filtering, and waste excretion (Guimaraes-Souza et al. 2009).

So far, kidney regeneration strategies have relied on the use of scaffolds to make grafts, especially those obtained from decellularized kidneys deemed not suitable for transplant (Orlando et al. 2013; Karczewski and Malkiewicz 2015; Moon et al. 2016). Injection of adipocyte derived stem cells, which are a source of mesenchymal stem cells, has shown some promise, as it resulted in reduced renal dysfunction and increased tubular regeneration after injury on a unilateral severe ischemia mouse model (Almeida et al. 2013). Other cell sources, such as primary kidney cells (Guimaraes-Souza et al. 2012), induced pluripotent stem cells, adult bone marrow, embryonic and placental stem cells, have also shown to be suitable to produce or differentiate into kidney structures, either by environment manipulation or seeding on scaffolds (Little 2006; Karczewski and Malkiewicz 2015).

Due to its structural complexity, scaffold-free approaches have not yet produced suitable in vitro models or kidney grafts adequate for tissue regeneration. However, a recent study has taken us one step closer to producing functional organoids. The authors have shown that it is possible to direct human embryonic stem cells or human induced pluripotent stem cells to differentiate into different renal structures in vitro, depending on the timing and duration of exposure to small molecules which modified the initial Wnt signal (GSK-3 inhibitor) or promoted the growth of specific cells (FGF-9) (Takasato et al. 2015). The concept itself is not new (Mae et al. 2013), but these organoids resemble human embryonic kidneys and are comprised of four

compartments, showing evidence of functional maturation: the collecting duct ($GATA3^+$, $ECAD^+$), distal tubule ($GATA3^-$, $ECAD^+$, LTL^-), proximal tubule ($ECAD^-$, LTL^+), and the glomerulus ($WT1^+$). This has allowed not only the production of a more realistic model but also helped to elucidate these events during embryogenesis (Takasato et al. 2015).

7.3 Liver Regeneration

Similarly to kidney, extracellular matrix scaffolding provided by decellularization is now also possible for liver. The material is amenable to repopulation by hepatic cells and is not rejected when implanted into mice (Mazza et al. 2015). Although promising, these strategies rely on the availability of actual livers and potential problems such as accidental disease transmission may arise. Manipulation of the extracellular matrix during the decellularization process may also cause alterations that result in mis-repopulation or mis-differentiation once implanted in vivo or when used for in vitro studies.

Scaffold-free approaches have also been developed. Primary hepatocytes were cultured on temperature-responsive poly(*N*-isopropylacrylamide)-coated plates and used to produce cell sheets. These were treated with basic fibroblast growth factor (to stimulate neovascularization) and transplanted into the subcutaneous space of mice, where they remained well integrated for over 200 days. Furthermore, the tissue showed hepatic functionality and histological analysis of the 3D organization of sheets revealed liver-like appearance (Ohashi et al. 2007).

A different methodology consists of transplanting hepatocytes encapsulated in hydrogels into rodent models of acute liver failure. Human hepatocyte beads surrounded by alginate were well tolerated, showed absence of immune cells on their surface, and contained viable and functional liver cells, while improving liver damage (Jitraruch et al. 2014). Microliver tissue encapsulated in collagen-alginate was implanted in 90% partial hepatectomized mice and promoted regeneration of the caudate lobe, as well as improved survival (80%, compared to 10% observed in the controls) (No da et al. 2014).

7.4 Periodontal Regeneration

Human periodontal ligament cells harvested from extracted teeth were used to prepare sheets that were transplanted onto a mesial dehiscence rat model. Tissue regeneration has occurred and fibrils as well as an acellular cementum-like layer were present (Huang and Zhang 2011). The same type of cell sheets had previously been applied to dogs with surgically created dehiscence defects, where bone formation, periodontal ligament, and cementum were observed (Akizuki et al. 2005).

Regarding clinical trials using scaffold-free materials, there is one on www.clinicaltrials.gov (study ID NCT01814436) that is currently recruiting. Scaffold-free pellets of stem cells from human exfoliated deciduous teeth will be used to

assess safety and regenerative efficacy in patients with avulsed immature permanent teeth and pulp necrosis.

The same group of researchers has recently demonstrated that scaffold-free stem cell sheet-derived pellets from dental root apical papilla can form a vascularized pulp/dentine complex in empty root canals. The pellets contained extracellular matrix and exhibited higher expression of both bone and dentine sialoprotein, alkaline phosphatase, and runt-related gene 2 compared to traditional cell sheets, showing higher osteogenic potential (Na et al. 2016).

7.5 Cartilage

Cartilage regeneration still poses a challenge for both surgeons and researchers. Strategies to develop suitable solutions have spanned from cell-free approaches (Gille et al. 2010), to a mixture of cells and scaffolds to, more recently, scaffold-free methods.

Laryngotracheal reconstruction was attempted by using cartilage sheets made from autologous rabbit chondrocytes isolated from auricular cartilage. Although some signs of mechanical failure were observed, the structures were well integrated, did not elicit inflammatory reaction, and were covered in mucosal epithelium. This suggests that stronger constructs may be suitable for grafting (Gilpin et al. 2010).

A clinical trial, in Japan (study ID UMIN000017944), is also recruiting volunteers to evaluate the safety and osteochondral regeneration using implanted high-density mesenchymal scaffold-free stem cell autologous constructs derived from adipose tissue for donor site of mosaic plasty plugs. This follows on from encouraging preclinical studies where cartilage and subchondral bone regeneration were achieved in rabbits knees (Ishihara et al. 2014).

Most of the clinical trials currently underway using tissue engineering rely on the use of directly injected or applied stem cells or structures containing cells and scaffolds, with various degrees of predicted or demonstrated reabsorption. Search for the term “scaffold-free” has only retrieved one study, the abovementioned trial using pellets of stem cells from human exfoliated deciduous teeth. Cell sheets are being used in clinical trials for phase I, phase II, and phase III studies for a wide range of applications, such as healing of esophagus mucosal defects after endoscopic resection (NCT02455648), heart failure (for example, NCT02672163), articular cartilage defects (for example, NCT01694823), and ocular surface disease (NCT01123044).

8 Advantages and Challenges

In a similar fashion to their counterparts that use scaffolds, scaffold-free structures allow cell differentiation, cell-cell and cell-matrix interactions, remodeling, and assembly. It may be argued that, in fact, some of these processes can occur faster in scaffold-free structures, as there is no physical barrier to cell-cell communication,

cell and small molecules migration/diffusion and fusion. When a scaffold is present, time may be required for it to degrade, so that cells can move or make connections. These may be with host tissue in the course of integration or with each other during the maturation phase.

An exception to this may be the regeneration of periodontal tooth supporting structures that require selective wound healing and membranes are used to protect them from blood clots and connective tissue, which would interfere with the process (El Haddad et al. 2014).

Complex architectures and bigger structures may be more technically challenging to put together, in the absence of rigid templates with defined shapes. Tissue deformation, partly caused by tension on the actin cytoskeleton, can be reduced by the addition of Y-27632, a selective inhibitor of ROCK phosphorylation (Czajka et al. 2014). Although still a challenge, studies that specifically look at this problem and how to increase intrinsic resistance, as well as the development of creative solutions where a scaffold or a mold can provide support but does not become an integral part of the structure, may push the capabilities of scaffold-free tissue engineering further.

In some cases, scaffolds are able to encourage or direct differentiation and homing of different cells to distinct areas. Self-assembled structures must produce their own extracellular matrix and chemical cues in order to obtain the correct degree of cell differentiation and structural organization. Scaffold-free assemblage seems to rely on processes similar to those naturally occurring during development. The resulting microenvironment, without exogenous structures and barriers to cell-cell/cell-matrix interactions and migration brings the process closer to physiological conditions. Also, the degradation of scaffolds may hinder remodeling and integration, not only in terms of time but also in terms of release of undesirable compounds. Thus, scaffold-free structures have the potential to make the process faster and safer/more reliable. For example, the addition of enzymes or other molecules seems to contribute to the formation of new tissue (Responte et al. 2012). One drawback is the possible lack of knowledge of all the factors involved, but in recent years there has been an increasing body of new literature on developmental processes and cell (re) programming.

The absence of a physical barrier may also influence mechanotransduction, as it can begin immediately after assembly or transplantation. The presence of structures, however temporary they may be, with a stiffness different to what cells might encounter in an in vivo situation may have an influence on cell morphology and behavior beyond the life span of the scaffold. Studies have shown that cells grown on a certain surface, harvested and then seeded on a different surface are able to retain a memory of the previous conditions, affecting their future behavior.

The fact that scaffold-free structures are able to begin fusion and remodeling more promptly, as well as are more permeable to small signaling molecules than those containing scaffolds, may confer them an advantage in terms of graft survival in vivo, which is crucial for the success of surgical implantation. However, everything comes at a cost. Scaffold-based structures use less cells, so the process is slower, as they need to expand. Staring with high cell numbers is beneficial to

achieve conditions similar to those in nature, but cells are usually expensive both to buy and maintain.

An important aspect to consider when using scaffolds is the unknown effects of the scaffolding materials or their interactions with living cells. These can be chemical, thus altering normal signaling and cell behavior, as well as physical, as mentioned above. For pharmaceutical studies, unknown chemical interactions between the drugs and scaffold materials may occur that can result in incorrect data. The material itself may be toxic to the cells or its physical presence may interfere with cell migration or access to shear stress. From the point of view of transplantation of artificial engineered tissue into patients, scaffold-free structures may offer advantages in terms of not eliciting an inflammatory reaction, which causes graft death and rejection.

Structures that are made from cells only and their products, such as extracellular matrix, are cultured in aseptic conditions with no addition of exogenous materials, except for sterile media and supplements. This affords less risk of infection by microorganisms that may lead to septicemia.

The process of obtaining constructs itself is less punishing on the cells as they do not need to be exposed to so many steps, chemicals, compression, temperature, and manipulation. This results in higher cell viability and less overall disturbance in terms of signaling and behavior.

Cell sheets collected from thermoresponsive substrates do not require the use of enzymes for detachment, which may have negative effects on them. They will adhere to host tissue, eliminating the need for suturing or glue (Huang and Zhang 2011).

Both technologies have advantages and disadvantages, which may render them more or less appropriate, depending on the application. The increasing understanding of cellular and molecular processes, as well as developments in materials (both biological and nonbiological) and equipment, is shaping the way tissue engineering is done and progress will continue towards more realistic and effective therapies, as well as in vitro models.

9 Conclusions

Recent advances in the manufacturing industry, scaffold materials, and biodegradable hydrogels, as well as the understanding of developmental and cell biology, have led to the current rapid growth of 3D bioprinting and its applications. Two main approaches have emerged: those using scaffolds and scaffold-free processes. The definition of "scaffold-free" can be confusing at times, as temporary scaffolding may be used, but the final biological product should be scaffoldless at the time of implantation or testing.

Various scaffold-free systems have been developed, such as cell sheets (and variations), those using single cells as building blocks or spheroid aggregates. There is a selection of methods that can be employed to obtain spheroids but, after the cells aggregate, remodeling and extracellular matrix deposition will occur by self-assembly. This is similar to the natural processes occurring during development.

Seminal studies in this field, as well as more recent literature, have provided valuable insights into how cells are regulated by their microenvironment, namely which factors contribute to cell differentiation, migration, adhesion, coalescence, and interactions with other cells, molecules, and matrix.

Printing living cells to form tissue is more complex than printing plastic or metal objects. However, new biocompatible materials and bioprinting techniques are being developed, including those able to produce scaffold-free structures. These require a higher number of cells and are more difficult to obtain due to the lack of structural stiffness but have several advantages over their counterparts containing scaffolds. These include faster remodeling, extracellular matrix deposition and integration with host tissue if implanted, absence of potentially hazardous chemicals, as well as a physical barrier within the tissue.

Although complex structure, especially that of solid organs that comprise many different cell types and an intricate microvascular network is still a challenge, the multidisciplinary nature of the field allows for the development of innovative solutions that would take longer to achieve from a single point of view. Currently, there are several scaffold-free 3D bioprinted structures that are already being used for research and clinical applications throughout a variety of specialties, from the cardiovascular system to dentistry. Also, the number of ongoing and recruiting clinical trials using tissue created with this technology has been increasing in the past few years. Therefore, it is reasonable to expect this upwards trend to continue in the future, with more tissue-based therapies and in vitro tools being trialed and put into practical use.

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