In Vitro Biofabrication of Tissues and Organs

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INTRODUCTION

After the sensational images of the mouse growing a human ear were broadcast around the world in the late 1990s, the in vitro fabrication of tissues and the regeneration of internal organs were no longer regarded as science fiction but as possible remedies for the millions suffering from chronic degenerative diseases.

Although some mistook it as a genetically engineered mouse expressing a human ear [1], these striking images nonetheless highlighted the medical promise of "tissue engineering" and ignited widespread interest from researchers in many fields, including cell and molecular biology, biomedical engineering, transplant medicine, and organic chemistry.

While there have already been successful clinical reports documenting the treatment of severe burn patients with culture-expanded skin cell sheets since the introduction of this tissue engineering technology in 1981 [2], fabrication of three-dimensional (3D) tissue constructs in vitro remains a challenge.

In the above-mentioned study, Cao et al. prepared a biodegradable polymer scaffold in the shape of a human ear and seeded its surface with bovine chondrocytes. This "tissue engineered ear" was then implanted under the skin of a nude mouse. As nutrients were provided by the in vivo environment, the implanted chondrocytes gradually started producing extracellular matrix (ECM) components such as collagen and glycoproteins. While a cell-free ear-shaped polymer could not have maintained its original shape in vivo due to the hydrolytic degradation of the polymer, the chondrocytes seeded onto the polymer maintained the original scaffold shape for 12 weeks after implantation. Indeed, the geometry was similar to and as complex as the original human ear.

After the study of the mouse with the human ear, many researchers attempted to create tissues or organs in vitro by constructing scaffolds composed of various biocompatible materials, such as animal-derived collagen [3], synthetic polymers [4], artificially synthesized bone substitutes (calcium-phosphate cement) [5], and autologous fibrin glue [6]. These scaffolds were seeded with a large array of somatic cells or stem cells to reconstruct target tissues such as skin [7], bladder [8], articular cartilage [9], liver [10], bone [11], vascular vessels [12], and even a finger [13].

The combination of a scaffold with cells and/or growth factors became the gold standard of tissue engineering [14]. Successful application of scaffold-based tissue engineering depends on three steps: (1) finding a source of precursor or stem cells from the patient, usually through biopsy or isolated from accessible stem cell-rich tissues, (2) seeding these cells in vitro onto scaffold material of the desired shape (with or without growth factors) that promotes cell proliferation, and (3) surgically implanting the scaffold into the target (injured) tissue of the patient.

This tissue engineering method overcomes a number of problems associated with allogeneic organ transplantation: the perpetual shortage of donors, the possibility of rejection, ethical issues such as organ trafficking [15], and the need for prolonged immunosuppression, which may lead to opportunistic infections and increased risk of cancer [16].

Many researchers tried to fabricate organs by combining cells, proteins/genes, and scaffolds. The various biomaterials used to fabricate scaffolds are classified into three types: (1) porous materials composed of biodegradable polymers, such as polylactic acid, polyglycolic acid, hyaluronic acid, and various co-polymers; (2) hydroxyapatite or calcium phosphate−based materials; and (3) soft materials like collagens, fibrin, and various hydrogels and their combinations.

In addition to providing a 3D structure for transplanted cells, scaffolds also dramatically enhance cell viability (e.g., a few exogenous cells were detected after the transplantation of single isolated cells into infarcted myocardium [17,18]). Anchorage-dependent cells cannot survive for long when detached from the surrounding ECM or culture surface. When there is loss of normal cell−cell and cell−ECM interactions, unanchored cells may undergo a specific form of pro-grammed cell death called "anoikis" [19,20]. Thus, seeding anchorage-dependent cells onto scaffolds allows for efficient transplantation, especially if scaffolds are pretreated with growth factors. Indeed, some scaffold-based tissue engineered systems, such as bladder [21], articular cartilage [22], epidermis [23], and peripheral pulmonary arteries [24], have already been translated into the clinical stage.

1.1 Problems with scaffold-based tissue engineering

The ideal biodegradable scaffold polymer should be (1) nontoxic; (2) capable of maintaining mechanical integrity to allow tissue growth, differentiation, and integra-tion; (3) capable of controlled degradation; and (4) nonimmunogenic; also, it should not cause infection or a prion-like disease. Although there are many clinical reports on the successful use of various biomaterials, there is still no "ideal" biomaterial for scaffold construction. Furthermore, concerns such as immunogenicity, long-term safety of scaffold degradation products, and the risk of infection or transmission of disease, either directly or concomitant with biofilm formation, remain to be resolved.

1.1.1 Immune reactions

A serious concern is that scaffolds may induce undesirable immune reactions [25], including inflammation, acute allergic responses, or late-phase responses. Scaffolds might even stimulate an autoimmune response, such as that produced by type II collagen in mice [26−28] used as models for rheumatoid arthritis. Immune responses may also be triggered by scaffold degradation byproducts. Metallosis is a specific form of inflammation induced by tiny metal particles that are shed from the metallic components of medical implants, such as debris from artificial joint prostheses [29]. Accumulation of scaffold degradation byproducts may elicit chronic diseases associated with inflammatory responses.

1.1.2 Degradation of scaffolds in vivo

Classic biodegradable polymers are defined as materials that are gradually digested by environmental bacteria through a process that is distinct from physio-logical degradation processes like digestion. Biodegradation can lead to toxicity in two ways: either a degradation product is directly toxic or it is metabolized to a toxic product (i.e., by liver enzymes). "Biodegradable" is distinct from "biocompatible." In most industrialized countries, only certified biomaterials that

have passed multiple tests for severe toxicity and safety are permitted for use as medical implants.

Most synthesized biodegradable polymers are broken down by hydrolysis, resulting in the accumulation of acids that may alter the pH of the microenvironment or exert more direct toxicity. Some scaffolds are destroyed by macrophages, inducing an inflammatory reaction.

While bone substitute scaffolds may be replaced gradually by true bone through the activity of osteoclasts and osteoblasts, degradation of most other biomaterial scaffolds will leave a potential space that can impede repair. Biodegradable biomaterials are used extensively for cartilage repair, since articular cartilage (hyaline cartilage) has a low regenerative capacity and is usually replaced by weaker, rougher fibrous cartilage after injury [30]. When the scaffold is degraded and disappears, the space that once occupied it may no longer be filled with chondrocytes due to the cells' low proliferative capacity. These spaces might eventually form tiny cracks that trigger further deterioration of the smooth cartilage surface.

1.1.3 Risk of infection

There are two potential sources of infection from implanted scaffolds: pathogens transmitted directly from the scaffold or cells and infections emerging from the bacterial biofilm formed around the scaffolds after implantation.

1.1.3.1 Potential risk of disease transmission by scaffolds

Some scaffolds, such as collagen gels and amniotic membranes, are animal-derived. Recent outbreaks of severe infectious diseases like bovine spongiform encephalopathy and severe acute respiratory syndrome highlight the fact that animals harbor pathogens that may be lethal or cause severe infections in humans. Moreover, it is safe to assume that there are many undiscovered animal pathogens with the potential to cause human disease or death. Preclinical studies may minimize this risk, but there is no guarantee that these materials do not harbor unknown human pathogens.

1.1.3.2 Biofilms

Another source of infection from implanted scaffolds is the biofilm that forms on the scaffold surface [31]. Medical devices and implants, such as catheters and orthopedic or dental implants, are now ubiquitous in clinical practice. However, as the number of devices and implants continues to increase, the frequency of device-related infections will also increase [32,33]. Infections that are mostly caused by staphylococci, such as methicillin-resistant *Staphylococcus aureus*, usually do not respond to antibiotic therapy, necessitating removal of the implanted device.

In vivo microbial contamination of these devices differs from infection of natural tissues. Medical devices lack an immune system or bloodstream. Thus, once

microorganisms invade through skin scratches, wounds, airways, or medical interventions and attach to the surface of the implanted material, they begin to form a bacterial biofilm [34]. The biofilm is composed of glycoproteins and polysaccharides secreted by microorganisms. Unlike circulating bacteria, biofilm-protected microorganisms are resistant to physical removal, host immunity, and antibiotics. Furthermore, since most antibiotics are unable to completely diffuse inside the biofilm, long-term antibiotic treatment may increase the risk of antibiotic resistance. In the United States, for example, catheter-related infections are a major cause of nosocomial morbidity and mortality. More than 300,000 U.S. patients are infected annually during presurgical or surgical procedures [35]. Moreover, as biofilms are slow to develop, infections due to biofilms may emerge several years after implantation. In artificial joint replacement surgery [36], this type of infection is a serious complication that can usually be cured only by removing the implant [32,37]. Infection by microorganisms is also widespread among contact lens users. One common cause of vision loss is contact lens−related microbial keratitis [38,39], and the risk of microbial keratitis increases during extended wear. This is why clinicians recommend frequent removal or replacement of contact lenses [38]. Furthermore, infection is the most common reason for breast implant removal [40,41]. These biofilm-related infections prolong hospitalization, increase medical costs, and sometimes result in mortality.

It is evident from the preceding discussion that scaffolds have several potential disadvantages. However, because there have been no clinical case reports documenting scaffold-related infection in regenerative medicine, many researchers have paid little attention to the possibility of infection from pathogens in the implant or biofilm.

Although most biomaterials used as scaffolds are biodegradable, degradation is usually very slow and may take several years. When infection occurs at the scaffold site, curing the infection may require surgical removal of the scaffold, disrupting tissue repair or causing further damage.

Various attempts have been made to develop infection-resistant biomaterials, such as silver ion−coated materials, ceramics that slowly release antibiotics [42], and antibacterial adhesion polymers [43], but it may take years before these materials are used in regenerative medicine, especially because these antibacterial factors may also harm the implanted cells. Thus, while scaffolds may hold great clinical potential, there remain significant safety concerns.

1.2 "Scaffold-free" tissue engineering

A precise definition of "scaffold-free" is still controversial [44]. Some investigators would insist that some of the techniques described below should not be called "scaffold-free" because the implanted construct may include residual biomaterials from the fabrication process. For the purpose of this section, a "scaffold-free"

system is a "cell-only" construct that may or may not use other biomaterials during fabrication. Even if it does contain other biomaterials, these are not implanted along with the cells.

From a clinical perspective, the most important property of a scaffold is its behavior in the body upon implantation (degradation, biofilm formation) and physiological reactions induced by the parent material and degradation byproducts (immune responses, local or systemic infections).

### 1.2.1 Classification of present scaffold-free systems

Several scaffold-free systems have been reported, some of which are already used for clinical treatments. These systems can be divided into three categories according to the cellular material used for construction. One system uses single cell sheets, another uses isolated single cells, and the third uses spheroid cell aggregates as the essential building blocks for implantable 3D constructs (Figure 1.1).

1.2.1.1 Cell sheets

Cell sheet technology is one of the most advanced methods for the construction of implantable engineered tissue. Certain types of cells can be removed from a culture dish as a relatively stable confluent monolayer-sheet [45]. Cell sheet technology is already used clinically for the repair of skin [2], cornea [46], esophagus [47], heart muscle [48], and blood vessels [49], and it is a promising method for many other applications in tissue engineering and regenerative medicine.

The first successful clinical application of cell sheets was developed by Rheinwald and Green [45] to treat patients with severe burns. At that time, keratinocytes were difficult to culture for expansion. Rheinwald et al. seeded a suspension of disaggregated keratinocytes onto a feeder layer of irradiated mouse 3T3 cells. The feeder layer enhanced plating efficiency and stimulated keratinocyte proliferation. Proliferation and culture life span could be further increased by adding various supplements or growth factors to the culture medium. They were able to recover single continuous sheets of keratinocytes that could be grafted onto the sites of severe burns. Many patients with severe burns have survived due to this skin sheet technology [7]. Since then, grafting of these keratinocyte monolayers is perhaps the most successful example of tissue engineering therapy, and several products have been examined in clinical trials. A number of them have been approved by the FDA and are now on the market [50].

In January 2009, Japan Tissue Engineering Co., Ltd., a Japanese biotechnology company, began marketing autologous cultured epidermis (called JACE) as the first Japanese tissue engineering product covered by national health insurance. JACE uses Green's [45] cell sheet engineering system, and it is the only regenerative medicine product currently approved by the Japanese Ministry of Health, Labor and Welfare [51]. This approval is significant because the Japanese MHLW was considered to be the utmost conservative authority for the approval
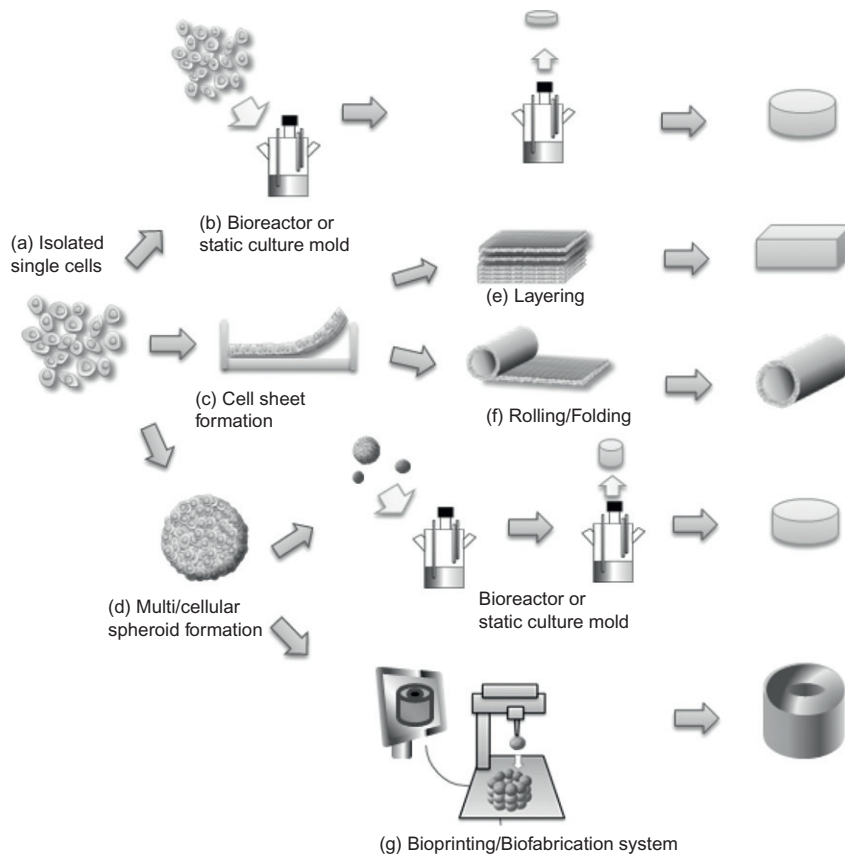
FIGURE 1.1

Methods for scaffold-free biofabrication. (a) Isolated single cell suspension. (b) Culture isolated cells in bioreactor or in static culture mold. (c) Cell sheet formation. (d) Multicellular cellular spheroid formation. (e) Layering cell sheets. (f) Rolling or folding cell sheets. (g) Computer-based bio-printing/biofabrication system.

of new drugs and medical devices and thus may indicate more timely approval and acceptance of similar products in Japan and elsewhere.

Okano et al. developed an alternative method for cell sheet engineering by first coating culture dishes with a temperature-responsive polymer, poly(N-isopropylacrylamide) [52]. This surface is relatively hydrophobic and similar to standard culture dishes at 37°C, but it becomes hydrophilic below 32°C. Various cell types can attach to the surface and proliferate at 37°C, while cooling below 32°C causes the cells to detach without the use of enzyme digestion reagent [53]. This is in contrast to Green's [45] cell sheet method, which always requires

dispase for recovery of cell sheets from culture dishes. The method of Okano allows the production of many types of cell sheets that are too fragile or otherwise difficult to recover by other methods [53−55]. Furthermore, Okano's method does not require an earlier used exogenous feeder layer, thus representing a potentially safer method. (Earlier employed feeder layers containing mouse 3T3 cells produce mouse proteins that may induce allergic reactions.)

1.2.1.1.1 Corneal sheets
The clinically most advanced application of the system developed by Okano is corneal regeneration using cultivated human corneal sheet transplantation [46]. Kinoshita et al. also showed good clinical results with cultivated human corneal sheet transplantation [56]. However, their system is not scaffold-free by our definition because it used allogeneic amniotic human membrane as an autologous cell carrier. Nishida et al. harvested corneal epithelial stem cells from the limbus of patients with severe ocular trauma, such as alkali burns, or ocular diseases, including autoimmune disorders or Stevens-Johnson syndrome (erythema multiforme). After monolayer expansion in vitro, the corneal epithelial stem cells were formed into cell sheets using Okano's thermal responsive culture plates. Harvesting and transplantation of noninvasive cell sheets using this temperature-responsive culture system has also been applied for ocular surface regeneration.

1.2.1.1.2 Heart regeneration
Using Okano's method, Sawa et al. implanted a cultured skeletal muscle cell sheet into the damaged heart of a patient with degenerative cardiomyopathy, a disease characterized by progressive heart failure [48]. The patient was at end-stage heart failure and on life support using a mechanical left ventricle assisting system. The implanted cells were isolated from an approximately 10-g piece of skeletal muscle excised from the medial vastus muscle under general anesthesia. After monolayer expansion, 20 skeletal myoblast cell sheets were obtained and autologously implanted onto the patient's dilated heart through left lateral thoracotomy. Seven months after implantation, the patient was discharged from the hospital and no longer required artificial heart support.

1.2.1.1.3 Esophageal ulcer treatment
With the rapid progress of endoscopy, large esophageal cancers can be removed by a single procedure, such as endoscopic submucosal dissection (ESD). Endoscopic resection has become the standard treatment for esophageal lesions, replacing longer open surgery procedures. However, massive resection of esophageal cancer by ESD can be complicated by postoperative inflammation and stenosis (narrowing of the esophagus). Severe inflammation causes esophageal scarring, while stenosis may make swallowing difficult and painful. Although treatment with balloon dilation or temporary stents can enlarge the narrowed esophagus and partially and temporarily overcome this problem, an extended

response generally requires repeated treatment that can lead to further inflammation and postoperative pain.

Postoperative inflammation and stenosis are caused mainly by massive ulceration of the esophageal surface. Following a successful preclinical trial in dogs, Ohki et al. performed clinical trials using cell sheets to treat large esophageal ulcers [57]. To this end, they developed a device that can directly transfer and attach cell sheets through endoscopy without suturing. The application of these epithelial cell layers enhanced wound healing and reduced host inflammatory responses without causing stenosis.

1.2.1.1.4 Blood vessels

Good clinical results were obtained when cell sheet−based scaffold-free blood vessels were used for the facilitation of hemodialysis (HD) treatment [12,58]. These vessels were fabricated by wrapping a dehydrated fibroblast sheet around a polytetrafluoroethylene (PTFE) tube cylinder and then overlaying a living smooth muscle cell sheet and an outer fibroblast sheet. After culturing this multilayered "cell roll," the PTFE cylinder was removed and the lumenal surface was seeded with endothelial cells. A small clinical trial was conducted using this scaffold-free tube in ten patients with end-stage renal failure treated by HD through an arteriovenous fistula (shunt). Patients who require HD always face shunt complications such as infection and low blood flow due to clotting. L'Heureux et al. fabricated tissue engineered blood vessels with autologous cells from each patient and implanted the vessel as a replacement HD shunt. The implanted grafts were stable in vivo for 3 months and withstood repeated puncture for HD for up to 13 months, allowing uninterrupted HD [59].

1.2.1.1.5 Nerve grafts

Baltich et al. fabricated scaffold-free tubular constructs consisting of an external fibroblast layer and an internal core of interconnected neuronal cells derived from fetal rat spine. The conduction velocity of this engineered "nerve" was comparable to that of the sciatic nerve of a 4-week-old rat and approximately 50 percent of that observed in a 12-week-old (adult) rat [60]. These results suggest that the scaffold-free nerve grafts may be useful for peripheral nerve repair.

1.2.1.1.6 Liver regeneration

Fabrication of liver tissue in vitro has attracted considerable interest given the innate regenerative capacity of the liver and prevalence of liver diseases. Ohashi et al. [10] fabricated hepatocyte cell sheets by culturing hepatocytes on temperature-responsive poly(*N*-isopropylacrylamide)-coated culture dishes. Sheets of hepatic tissue transplanted ectopically into the subcutaneous space were pretreated with growth factor FGF-2 to promote neovascularization. These sheets efficiently integrated with the surrounding tissue and persisted for longer than 200 days. The engineered and implanted hepatic tissues also showed several characteristics of liver-specific functionality. Furthermore, layered hepatic tissue

sheets reorganized into a 3D structure with the histological appearance of liver tissue [10].

1.2.1.1.7 Implantation of pancreatic islets

The observed functional differentiation of ectopically implanted hepatocytes led Ohashi and Okano to perform a similar experiment using cell sheets composed of rat pancreatic islet cells [61]. In vitro, these pancreatic islet cell sheets retained the functional activity of native islet cells, including the production of insulin and glucagon, and glucose-dependent insulin secretion. Moreover, when transplanted into the subcutaneous space of rats, pancreatic islet cell sheets produced and secreted insulin, suggesting a new therapeutic approach for the treatment of diabetes mellitus and other diseases involving dysfunctional islet cells and possible elimination of the need for daily insulin injections [62,63].

The Okano group is now aggressively expanding potential applications by developing sheets for regeneration of bone [64], articular cartilage surface [65], periodontal ligament [66], lung [67], thyroid [68], and bladder [69].

1.2.1.1.8 Expansion of cell sheets into 3D structures

Various approaches have been used to fabricate larger 3D tissues and organs from cell sheets. One example is the "Origami" approach, where, like L'Heureux et al. [12], sheets are formed around a temporary 3D scaffold (like surgical tubing). Another standard approach is the layering of multiple cell sheets. Shimizu et al. layered beating cardiomyocyte sheets to fabricate scaffold-free 3D constructs and found that these layered cardiomyocytes exhibited synchronized beating. However, the maximum thickness was limited to less than $80\,\mu m$ (three-cell layer), possibly due to starvation and hypoxia of inner layers that have poor access to the culture media and atmosphere. Moreover, cardiomyocytes are tightly interconnected by gap junctions, and the outer layer may prevent gas and nutrient exchange to the inner layers. Indeed, four-layered cardiomyocyte constructs showed necrosis in the inner layers [70]. To overcome this limited maximum thickness, they implanted 10 three-layered cardiomyocyte sheets into nude rat hearts at 1- or 2-day intervals, finally obtaining a 1-mm-thick neomyocardium fused onto the native heart and integrated with a well-organized microvascular network. Although it is obviously impossible to perform multiple thoracotomies on human patients, this demonstration revealed the importance of neovascularization for the gradual construction of larger cell constructs with or without the use of scaffolds [71,72].

1.2.2 In vitro self-produced ECM-rich scaffold-free constructs

Certain cell types possess the capacity to synthesize and release components of ECM in vitro under appropriate culture conditions. Fibroblasts and chondrocytes, for example, produce collagens and proteoglycans in vitro. This ECM production capacity is accelerated under confluence or 3D culture conditions, a phenomenon

that may be inspired to develop scaffold-free systems for fabrication of 3D constructs from isolated chondrocytes [73,74]. Normal anchorage-dependent cells proliferate at low density in monolayer culture. When the cell density reaches a certain threshold, proliferation is suppressed by contact inhibition. Under contact inhibition, the cell cycle stops and chondrocytes start to produce ECM proteins. Using this in vitro−produced ECM, many groups have developed methods to fabricate 3D scaffold-free constructs in vitro. Most of these approaches are used for fabrication of cartilage-like constructs [75,76]. In this technique, a large number of isolated chondrocytes is loaded into a specific culture mold and fed reagents that enhance matrix production. Since chondrocytes exist under relatively low oxygen partial pressure and without blood supply in vivo (in joints), they are relatively harder than normal cells and can be cultured under high-density static culture conditions. Although these approaches require relatively longer culture, the resulting constructs are similar to native cartilage in terms of histology and biomechanical properties [77−79]. However, these "in vitro self-produced ECM-rich scaffold-free" constructs have limitations as well. It is difficult to expand cultures in 3D without hypoxia or nutrient starvation of inner core cells. Thus, most of these scaffold-free cartilage-like constructs are thinner than normal human articular cartilage in adults.

1.2.3 The rotating wall vessel bioreactor system

Another approach for fabricating scaffold-free constructs from isolated cells is by using a rotating wall vessel bioreactor system. This culture system utilizes a circular vessel with a gas-exchange membrane and rotates around a horizontal axis to provide culture media flow in a simulated microgravity environment. The rotating wall reactor (RWR) was developed by NASA to produce cartilage-like tissue in space [80]. This reactor has also been used on earth to fabricate various other cell constructs [81−83]. Okamura et al. loaded isolated hepatocytes into an RWR and obtained a "liver-like" construct with bile duct− and vessel-like structures formed within the tissue. Histological analysis showed that the bile duct structures secreted mucin and formed complex tubular branches in the peripheral region. Distal to these bile duct structures, they observed mature hepatocytes capable of producing albumin and storing glycogen [84]. To our knowledge, there are still no clinical reports using engineered tissue fabricated by this method.

1.3 Aggregation/spheroid-based approaches

The capacity of dissociated cells to reaggregate through cell−cell attachment has been known for over 100 years [85]. This phenomenon is preserved in almost all living organisms irrespective of their complexity [86,87]. These aggregates are usually called multicellular spheroids (MCSs) and are powerful research tools in

modern developmental biology, stem cell biology, tumor biology, toxicology, and pharmacology.

1.3.1 Preparation of multicellular spheroids

Although several methods for MCS preparation have been introduced, they all rely on a simple common principle: dissociated cells are incubated in a nonadhesive environment to allow individual cells to attach to one another. This cell—cell attachment is a survival mechanism that allows cells to avoid anoikis, possibly by activating signals mediated by surface receptors and ligands that suppress the anoikis cascade.

These MCSs can be prepared in regular nonadhesive culture dishes, silicon-coated dishes [88], containers coated with nonadhesive enhanced polymers (such as PDMS [89]) or poly-HEMA [90], agarose gels [91], alginate beads [92], spinner flasks [78], or hanging drop cultures [93]. After reaggregation of dissociated cells, each MCS has the capacity to fuse with other MCSs. Many groups have developed alternative approaches for scaffold-free tissue engineering using this propensity for MCS fusion. In fact, methods using spheroids as building blocks for fabrication of scaffold-free cell constructs may be a better approach because many cells in spheroids show greater similarity to cells in the native state than do cells in monolayer culture [94].

MCS fusion usually requires 24 to 72 hours, depending on the cell type and culture conditions. During fusion, these MCSs must be kept in culture media under controlled conditions because even a slight tilting of the culture dish may deform the desired shape of the stacked MCS blocks.

1.3.2 Molding MCSs

Most of these MCS-based approaches also use specific molding chambers to produce constructs of the desired shapes [91,95,96]. However, it remains difficult to fabricate larger tissues similar to the geometry of native tissues or organs due to limited gas and nutrient exchange within the core of the spheroids, particularly for hepatocytes, cardiomyocytes, and other cells with high nutritional or metabolic demands.

1.3.3 Bio-printing

Possibly inspired by common inkjet printers, cell printing systems, called bio-printing systems, have been developed [71,97]. Mironov, Forgacs, and colleagues first established a spheroid-based bio-printing system [98,99] that used MCSs as "bio-ink" and hydrogels as "bio-paper." Their printer lays down MCSs onto pre-designed spots on the hydrogel to allow adjacent MCSs to fuse until the desired shape is attained. Using this system, they fabricated a beating cardiomyocyte plate. Their latest system can print multicellular rods onto agarose (bio-paper)

using a dual-nozzle system for real-time molding of vascular and peripheral nerve constructs [100]. This bio-printing approach can fabricate more complex three-dimensional designs with microchannels that may allow for better in vitro perfusion and provide conduits for neovasculization in vivo.

1.3.4 Alternative approach for MCS assembly technique for biofabrication

We developed another approach for a scaffold-free MCS assembling system called a "needle-array" system (Figure 1.2) that is slightly different from bio-printing systems. Instead of using hydrogel as the "bio-paper," we used medical-grade stainless needles as temporal fixators to skewer MCSs until they fused with one another. This concept was inspired from surgical treatments for bone fracture in orthopedic surgery, called "external fixation" (Figure 1.3). For treatment of bone fracture, orthopedic surgeons reposition fractured bone pieces to their original positions with or without surgery. After repositioning, surgeons immobilize bone pieces by using casts or splints without surgery or by using metal plates, screws, or pins under surgical procedure. Inspired from fracture treatment, especially by the external fixation technique, we developed the needle-array
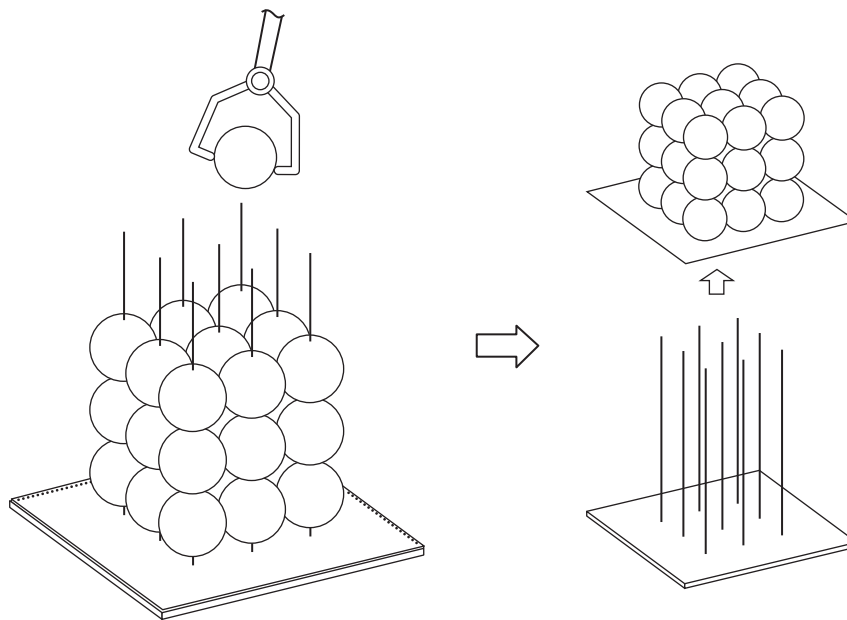
FIGURE 1.2

The needle-array system. (Left) Skewering a spheroid into the needle-array with a robotic system. (Right) Removing fused spheroids to obtain a scaffold-free construct.
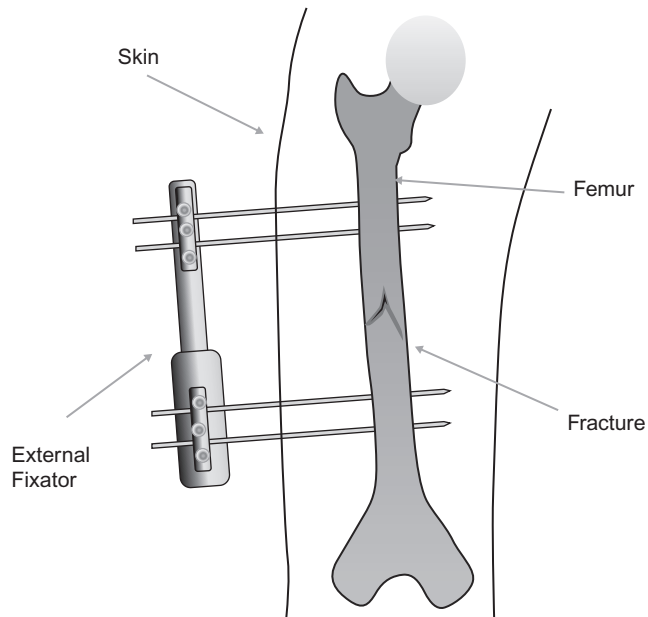
FIGURE 1.3

External fixation for bone fracture treatment. Pins are inserted through the skin into the bone.

system. We also developed a robotic system that skewers MCSs into needle-array according to a three-dimensional design template (Figure 1.4).

By applying these systems, we can fabricate complex three-dimensional scaffold-free cell constructs by using various types of cells such as chondrocyte, hepatocyte, cardiomyocyte, vascular smooth muscle cell, and so on. Since we utilize medical grade needles, it is easy to remove the temporary supports without contamination with exogenous materials. In addition, efficient gas and nutrition exchange could be expected, unlike in other scaffold-free MCS-based systems.

CONCLUSION

In this section, we reviewed the pros and cons of tissue engineering using scaffolds for regeneration of damaged tissue and discussed current developments in scaffold-free systems, some of which have already found clinical applications. We possess a large array of tissue engineering/regenerative medicine systems that must now be refined for clinical applications. Some of these systems show remarkable results in vitro but are challenging to translate into bedside.

FIGURE 1.4

Needle-array-based scaffold-free biofabrication system. (Left) DNA-like double helix design template. (Middle) 3D biofabrication system in a bio-clean bench. (Right) Scaffold-free endothelial cell—based construct based on the design template in the left pane. Scale bar = 1 mm.

The industrial giant Dow Corning, the largest supplier of silicone breast implants, filed for Chapter 11 bankruptcy in 1995 in the face of over 20,000 law-suits claiming that its implants caused systemic health problems, despite the fact that there was no direct evidence linking the implants to these health problems [101]. The lesson from this case is that despite promising results in vitro and in preclinical models (that are usually only monitored for a few months or years), the safety of scaffolds and implants must be confirmed in humans over the long term. Scaffold-free systems are an alternative for tissue engineering and repair of damaged tissues that may circumvent at least some of these potential risks.

Tissue engineering and regenerative medicine are under constant development, so it is too early to determine which is better: scaffold or scaffold-free engineered tissues. Whichever approach is used, to fabricate human-scale tissues or organs in vitro, new methods must allow for neovascularization to overcome the diffusion limits of oxygen and nutrients within tissues [71,72]. Endothelial cells have the capacity to form tubes and networks in vitro under appropriate culture conditions [102], so there is hope that the problems of neovascularization can be solved without the need for fabricating complex vascular networks in vitro.

In light of the latest developments on decellularized organs [103,104], it can be surmised that it will soon be possible to fabricate whole tissues and organs in vitro using the appropriate combination of cells, culture conditions, and bioreactors without the use of artificial scaffolds. To achieve the translation of these emerging tissue engineering technologies from benchside to bedside, with or without a scaffold, the safety, efficacy, and cost-effectiveness of the approach will have to be evaluated at each phase of clinical development.

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