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Additive Manufacturing of Tissues and Organs

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Abstract

Additive manufacturing techniques offer the potential to fabricate organized tissue constructs to repair or replace damaged or diseased human tissues and organs. Using these techniques, spatial variations of cells along multiple axes with high geometric complexity in combination with different biomaterials can be generated. The level of control offered by these computer-controlled technologies to design and fabricate tissues will accelerate our understanding of the governing factors of tissue formation and function. Moreover, it will provide a valuable tool to study the effect of anatomy on graft performance. In this review, we discuss the rationale for engineering tissues and organs by combining computer-aided design with additive manufacturing technologies that encompass the simultaneous deposition of cells and materials. Current strategies are presented, particularly with respect to limitations due to the lack of suitable polymers, and requirements to move the current concepts to practical application.

Keywords: additive manufacturing; bioprinting; biofabrication; hydrogels; tissue engineering

Contents

Nomenclature

2PP	two-photon polymerization
AM	additive manufacturing
BLP	biolaserprinting
CAD	computer-aided design
CT	computed tomography
DA	diacrylate
DMD	digital mirror device
ECM	extracellular matrix
FDM	fused deposition modeling
HA	hyaluronic acid
HEMA	hydroxyethyl methacrylate
LCST	lower critical solution temperature
MA	methacrylate
MMP	matrix metalloproteinases
NIPAAm	N-isopropylacrylamide
PEG	poly(ethylene glycol)
PPO	poly(propylene oxide)
RP	rapid prototyping
SFF	solid freeform fabrication
SLA	stereolithography (apparatus)
SLS	selective laser sintering
SPECT	single photon emission CT
STL	standard tessellation language
TEC	tissue engineered construct

1. The rationale

The fundamental concept underlying tissue engineering is to combine a scaffold or matrix, with living cells, and/or biologically active molecules to form a tissue engineering construct (TEC) to promote the repair and/or regeneration of tissues. The scaffold (a cellular solid support structure comprising an interconnected pore network) or matrix (often a hydrogel in which cells can be encapsulated) is expected to perform various functions, including the support of cell colonization, migration, growth and differentiation. Further, for their design physicochemical properties, morphology and degradation kinetics need to be considered. External size and shape of the construct are of importance, particularly if it is customized for an individual patient [1]. Besides the physical properties of a scaffold or matrix material (*e.g.* stiffness, strength, surface chemistry, degradation kinetics), the micro-architecture of the constructs is of great importance for the tissue formation process [2]. In recent years, a number of automated fabrication methods have been employed to create scaffolds with well-defined architectures [3, 4]. These have been classified as rapid prototyping (RP) technologies, solid freeform fabrication (SFF) techniques, or according to the latest ASTM standards, additive manufacturing (AM) techniques [5]. With AM techniques, scaffolds with precise geometries can be prepared [6], using computer-aided design combined with medical imaging techniques to make anatomically shaped implants [7]. Together with the development of biomaterials suitable for these techniques, the automated fabrication of scaffolds with tunable, reproducible and mathematically predictable physical properties has become a fast-developing research area.

The last few years have seen an upturn in economic activity and successful application of newly developed tissue engineering products, which for the largest part has resulted from

identification of products that are translatable from bench to bedside with available technology and under existing regulatory guidelines [8]. Cell-free scaffolds have shown clinical success, *e.g.* for bone (Fig. 1), osteochondral tissue repair, cartilage and skin [9]. Also, strategies to create new vasculature - a critical aspect of tissue engineering - are being developed by making use of the body's self-healing capacity [10].

Figure. 1.

Nevertheless, *cell-based* therapeutics have largely failed from both a clinical and financial perspective [12, 13]. The developed tissue engineering products were not necessarily inferior to previous alternatives, but the efficacy and efficiency were not sufficient to justify the associated increases in costs [14,15]. Manual cell seeding and culturing of pre-fabricated scaffolds is time-consuming, user-dependent, semi-efficient and, therefore, economically and logistically not feasible to achieve clinical application at an economical scale [16, 17]. Particular shortcomings of the current tissue engineering paradigm involving cell seeding of pre-fabricated scaffolds are the inabilities to:

- mimic the cellular organization of natural tissues
- upscale to (economically feasible) clinical application
- address the issue of vascularization

The use of additive tissue manufacturing addresses these points by the incorporation of cells into a computer-controlled fabrication process, thus creating living cell/material constructs rather than cell-free scaffolds (Fig. 2). The fundamental premise of computer-controlled tissue fabrication is that tissue formation can be directed by the spatial

placement of cells themselves (and their extracellular matrix), rather than by the spatial architecture of a solid support structure alone. Although still at an early stage of concept development and proof-of-principle experiments, it appears that endeavors following this approach are the most promising to deliver clinical solutions on the longer term where cell-free approaches cannot. Automated tissue assembly opens up a route to scalable and reproducible mass production of tissue precursors [18]. Furthermore, implementing good manufacturing practices (GMP), quality control and legislation are facilitated by the use of automated processes.

The aim of this comprehensive review article is to discuss current strategies of AM-related tissue engineering applications, particularly with respect to limitations due to the lack of suitable polymers and requirements, to move the current concepts to practical application.

Figure. 2.

2. Historical overview

In the classical picture of manufacturing, objects can be produced either tailor-made on a one-by-one basis, or by mass production. Mass-produced goods are much cheaper than tailor-made products that usually involve skilled manual labor, yet leave little room for customer specifications or requirements. With the advent of AM, this classical picture has started to change. AM enables engineers to create objects from personalized specific computer-aided designs, while employing automated processes and standardized materials as building blocks. Currently, AM technology is still quite expensive for the personal user groups, therefore, the fabrication of self-designed objects is mostly outsourced to

companies, but with fast-developing projects such as Fab@Home [19] it is realistic that in a decade from now many households will have their personal AM equipment. As a 3D analogue to inkjet and laser printers, this will allow users to fabricate personally designed objects in an inexpensive and automated manner.

With respect to medical implants, patients might have individual needs, based on specific anatomy or the possibility to include autologous cells to enhance the treatment. The combination of automation and flexibility in design is what makes AM very suitable for the generation of such personalized implants and devices. The behavior of cells can be directed by tailoring their environment. Patterning technologies can control surface chemistry and topography at scales smaller than a single cell. They can be designed to mimic the natural surroundings and regulatory microenvironments of cells *in vivo*, or to modify the microenvironment to study the cellular response [20-22]. In two dimensions, one has more control over the chemical and physical properties on a small scale, and imaging and characterization are simpler. Although a significant body of knowledge on cell behavior has been accumulated using patterned surfaces, two-dimensional (2D) techniques have been shown to be insufficient for some new challenges of cell biology and biochemistry, as well as in pharmaceutical assays [23]. The importance of a three-dimensional (3D) structure for *in vitro* experiments has been demonstrated by a number of studies [24]. For example, hepatocytes retain many of their liver-specific functions for weeks in culture in-between two layers of collagen gel, whereas they lose many of these functions within a few days when cultured as a monolayer on the same gel [25]. Also, it has been long known that chondrocytes retain their phenotype in 3D cultures, whereas they dedifferentiate when cultured on flat surfaces [26]. The vascularization of tissue-engineered bone is only possible

in 3D [27]. The current challenge is to improve 3D tissue manufacture techniques to a higher level of control at higher accuracies, aiming to recreate the *in vivo* niche with automated fabrication methods while retaining a clinically relevant production rate.

A time line starting from the invention of the first printing techniques up to the current state-of-the art in AM of TECs is graphically illustrated in Fig. 3 (additional relevant breakthroughs in science and technology have also been listed/included). Although automated processes dealing with cells, peptides and biomaterials have been around for nearly half a century (*e.g.* the first automated cell sorter was invented already in 1965), the first reported attempts to manufacture biological constructs including living cells dates back less than a decade. Pioneering work in this kind of printing was done in the Boland laboratory, using a simple home-office desktop printer with only minor modifications to deposit cells and proteins [28]. Inkjet printing has since then been studied and developed to a quite well-understood process capable of patterning viable cells and biomaterials [29]. A number of AM techniques have been developed or modified to include cells in the fabrication process, among which biolaserprinting [39, 31] (since 2004), stereolithography [32-35] (since 2004) and robotic dispensing [36-44] (which is based on fused deposition modeling and also referred to as 3D fiber plotting (3DF) or bioplotting) (since 2005). Recently, the very first use of AM directly *in vivo* was reported (biolaserprinting, 2010) [45]. Further, the exponential growth of this new field is illustrated by the establishment of the journal *Biofabrication* in 2009 and the International Society for Biofabrication (www.biofabricationsociety.org) in 2010.

Figure. 3.

3. State-of-the-art

It should be understood that, technologically, additive tissue manufacture is still in its infancy. Hydrogel structures containing viable cells have been produced, but the designs have been simple and isotropic, the dimensions have been limited to a few millimeters and the imposed requirement for mechanical properties has been 'self-supporting' or 'handleable'. Fig. 4 shows the results of some of the most advanced attempts to fabricate living constructs of cells and hydrogels with automated processes. These data sets show the potential of AM, yet at the same time the limitations and the embryonic stage of its development.

Figure. 4.

3.1. 2D patterning and direct cell manipulation

In vitro 3D models based on engineered human tissues are now emerging as a viable alternative to 2D cell culture assays (which often give false predictions due to an oversimplified cell environment) and *in vivo* experiments (which do not necessarily capture the important aspects of the human condition, and are limited in the possibility of environmental control). Nevertheless, some tissue manufacture techniques such as inkjet printing and biolaserprinting have emerged from technologies that initially aimed at the manufacturing of 2D systems. Here, we briefly discuss patterning and cell manipulation techniques that have been performed in 2D.

Working in 2D has several specific advantages. One has more control over the chemical and physical properties on a smaller scale, and characterization (particularly imaging) is easier.

Using patterning technologies, one can spatially control surface chemistry and topography at the micrometer level or even below. Most 2D patterning techniques involve the fabrication of a patterned surface by photo-lithography, followed by the selective functionalization of the patterned surface with for example cell-adhesive peptides, cell-repellent polymers or bound signaling molecules. Photolithography provides the unique ability to study cell-substrate interactions on single cells in confined areas. A specific disadvantage of photolithography is the high cost associated with the equipment and cleanroom facilities. Soft lithography uses elastomeric ('soft') stamps by casting and curing an elastomer (typically poly(dimethoxy siloxane)) on a silicon master [48]. With these stamps patterns of virtually any compound (including proteins) can be transferred onto most surfaces, without the use of UV or organic solvents. Using this collection of techniques, surfaces have been designed that mimicked the natural surroundings and regulatory micro-environments of cells *in vivo*, and micro-environment have been modified to study the cellular response [20-22]. Elastomeric stamps have even been employed to directly pattern living cells.

Another technique that allows direct manipulation of cells is laser-guided direct writing. Individual cells in suspension are guided (based on differences in refractive indices) by directed laser-light ('optical tweezers') to be deposited onto solid surfaces [49]. The cell-by-cell deposition theoretically allows the generation of precise patterns of cells, inducing specific cell-cell contacts. Furthermore, technologically simpler and less expensive alternatives for cleanroom-based photolithography are being developed. These mostly have lower accuracies, but still high enough to engineer an environment on the cell-size level. Examples are LCD-based projection photolithography [50] and transparency-based

lithography, where masks are obtained by simply printing patterns onto overhead projector sheets with a high-resolution office printer [51].

Surface patterning and direct cell manipulation techniques have proven to be useful tools to study direct cell-material interactions and we conclude that those will remain to be applied for this specific purpose. However, the designed micro-environments ultimately need to be expanded into the third dimension to be useful for the manufacturing of tissues and organs.

3.2. Additive manufacturing techniques

With AM techniques, objects from 3D model data sets can be constructed by joining material in a layer-by-layer fashion, as opposed to a subtractive manner in which most traditional manufacturing methodologies operate. In terms of tissue and organ manufacturing, the additive nature ensures minimal waste of scarce and expensive building material, namely cells, growth factors and biomaterials. The use of 3D model data enables fabrication of customized tissues, which is a *conditio sine qua non* for patient-specific treatment concepts. Further, AM techniques offer a high level of control over the architecture of the fabricated constructs, guarantee reproducibility and enable scale-up and standardization. The first step to produce a 3D object through AM is the generation of the corresponding computer model either by the aid of 3D CAD software or imported from 3D scanners [52]; there are a large number of imaging methods for data acquisition of human or animal body parts, such as X-ray computed tomography, magnetic resonance imaging, ultrasound echoscopy, single-photon gamma rays (SPECT) and bioluminescence imaging [53-56]. The CAD model is then tessellated as an STL file, which is currently the standard file for facetted models. Before manufacturing, the STL model is mathematically sliced into thin

layers (sliced model), which are reproduced into a physical 3D object by the AM device. Several well-developed and commercially available AM techniques have been employed to design and fabricate scaffolds for tissue engineering applications (Table 1).

Table 1 :

AM technologies produce 3D parts by spatially directed manipulation of materials in several possible ways: thermal, chemical, mechanical and/or optical. In thermal processes, the material is formed into an object after which it undergoes a thermal transition to fix the shape. In chemical-based processes, the manufactured shape is fixed by a chemical reaction (often polymerization). Mechanical processes rely on the physical deposition of cells or materials, and in optical processes cells or polymers are manipulated using light. Often several processing modes are combined in an AM technique (Table 2).

Table 2

In general, techniques that use optics can achieve the highest resolutions. Examples of accurate optical fabrication methods are stereolithography, laser direct writing and biolaserprinting. Additionally, photo-initiated polymerization can be used for safe encapsulation of cells and exogenous growth factors into hydrogels. Thermal techniques such as selective laser sintering or fused deposition modeling are not compatible with cells if requiring supra-physiological temperatures, but they can be adapted for processing thermosensitive hydrogels. Mechanical processes often allow for including cells in the fabrication process, as long as shear stresses induced on cells such as by deposition through a needle or inkjet cartridge orifice are sufficiently low.

Stereolithography is the oldest, most developed and most accurate of all AM technologies, and it has been applied for several biomedical applications including the fabrication of TECs with encapsulated living cells [68]. Although it is one of the few techniques with accuracies comparable to the size of a cell, its use has not been favored because to date, a system has not yet been developed that enables handling of different compositions of materials and/or cells. Pioneering work on tissue manufacture has been done using inkjet and laser printing. However, over the last few years the focus has been mostly on the robotic dispensing of hydrogels with encapsulated cells. With this class of techniques, highly viscous cell suspensions or liquid gel precursors are dispensed from cartridges or syringes through a nozzle and deposited as strands (Fig. 2). The method is versatile in terms of materials that can be used, in controlling the environmental conditions and in varieties of dispensing mechanisms (pneumatic, syringe pumps, extruder screws). The versatility and limited technological complexity are perhaps the main reasons for the relatively wide commercial availability of dispensing 'bioprinters'. A less-developed method that technologically could be applied to make living constructs in an automated manner is robotic assembly. High-precision robotic grippers can assemble pre-fabricated microscale building blocks into larger structures [78], and these building blocks could potentially be pre-seeded with different cell types. (a video of robotic assembly is available online as Supplementary Information).

3.3. Biomaterials

Over the last two decades, several biodegradable materials have been used and developed for the design and fabrication of scaffolds and matrices, including polymers (natural and synthetic) [79, 80], ceramics [81] and composites [82]. The polymeric and ceramic materials

that have been processed by AM to prepare scaffolds have all, with a few exceptions, been modified or synthesized specifically for use with a single AM technique, enabling accurate and reproducible fabrication of well-defined architectures with the anticipated physicochemical properties. However, these materials typically require process parameters (e.g., high temperature, solvents, lack of water) that are not conducive to direct inclusion of cells. Hydrogels are thus gaining increasing interest for the manufacturing of tissues [83].

3.3.1. Scaffold materials

Scaffolds for tissue engineering are mostly prepared from polymers, ceramics, or their combination (composites). To obtain an interconnected pore network many techniques have been employed including porogen leaching, gas foaming and phase-separation/freeze-drying. AM techniques however offer a higher degree of control over scaffold architecture [3], and a range of materials can be processed by AM techniques (Table 2). Stereolithography, the oldest and most developed of AM techniques, requires a photo-curable material. It has been employed to prepare scaffolds from poly(propylene fumarate) [84] and from (meth)acrylated poly(trimethylene carbonate co caprolactone) [85], poly(lactide) [86], polycaprolactone [87] and poly(ethylene glycol) [32-35], mostly in the presence of a diluent that can be either reactive or unreactive. Composites have been prepared by mixing in small ceramic particles in the stereolithography resin [88], and pure ceramic structures were realized by preparing composite structures with high ceramic loading, followed by burning out of the polymer while simultaneously sintering the ceramic [89].

Selective laser sintering has been used to prepare porous polycaprolactone (PCL) scaffolds, with or without additional calcium phosphate particles [76]. FDM-based tissue engineering

research has revolved around this polymer as well, leading to clinical application in the maxillofacial arena [11] and the establishment of bone tissue engineering concept based on a large long bone defect model in sheep [27]. 3DP has been applied to both synthetic and biopolymers (polylactide [65] and starch [66], respectively), as well as ceramics (hydroxyapatite [67]). Direct writing, a process similar to robotic deposition but at much higher resolutions achievable through to electrostatic interactions and coagulation, has been employed to fabricate well-defined silk fibroin scaffolds [72]. Although all mentioned materials are suitable for fabrication of scaffolds, the toxicity of their precursors or processing conditions often still does not allow the co-deposition of cells or cell-laden hydrogels in the manufacturing process. However, recent developments have shown a convergence of scaffold fabrication and cell deposition, combining the mechanical support of a scaffold structure with the automated and controlled placement of cells. These hybrid structures are discussed in detail in section 4.3.3. AM has proven its value for the preparation of scaffolds, and it is expected that current materials and processes will be adapted, and new ones will be put into place to allow the inclusion of cell-laden hydrogels in the fabrication process.

3.3.2 Hydrogels

Hydrogels are polymeric networks that absorb water while remaining insoluble and preserving their characteristic three-dimensional structure. This is because of the large number of physical or chemical links between the polymer chains. Hydrophilicity is one of the main factors that determine the biocompatibility of hydrogels, thus making them attractive for application in medicine and pharmacy as drug and cell carriers, and specifically for the design and fabrication of TECs [90].

As a result, they can provide embedded cells with a 3D environment similar to that in many natural tissues. Hydrogels are usually classified as either naturally-derived or synthetic. Naturally-derived gels (often derived from ECM itself) are generally good cell support materials, but have intrinsic problems, such as batch-to-batch variation, limited tunability and possibility of disease transfer. Synthetic hydrogels bear none of these disadvantages, but often lack biofunctionality. Besides these two classes of hydrogels, hybrid gels having both natural and synthetic components are gaining increased interest in tissue engineering, and more recently, in additive tissue manufacture. For example, naturally-derived hydrogels such as gelatin, hyaluronic acid and dextran have been functionalized with methacrylate or methacrylamide groups to enable (photo-initiated) cross-linking in combination with robotic dispensing [44, 91]. The methacrylate chemistry that was used here and before also for synthetic polymers, is versatile and can be applied to more naturally-derived hydrogels, including alginate [92]. The introduction of chemical cross-links at controlled densities not only enables fixation of printed shapes, but also allows tailoring of mechanical properties, swelling behavior, degradation kinetics and so forth. The chemical modification of naturally-derived hydrogels allows for combination of their intrinsic biofunctionality with the tunability of many properties through these synthetic components. On the other hand, synthetic gels are increasingly being functionalized with biologically active components such as cell-adhesive peptides, covalently bound growth factors, heparan sulphate, and protease-cleavable cross-links [93].

In additive tissue manufacturing, hydrogels are used both as a building material and as a cell delivery vehicle. Cells that have been viably encapsulated within hydrogels include fibroblasts, chondrocytes, hepatocytes, smooth muscle cells, adipocytes, neuronal cells and

stem cells [94]. During the AM of 3D tissue constructs, a hydrogel precursor solution with suspended cells needs to be processed into a defined, designed shape that is subsequently fixed by gelation. Therefore, the viscosity of the suspension needs to be sufficiently high to overcome surface tension-driven droplet formation, to enable drawing of thin strands of material, *i.e.* create well-defined shapes, and to prevent cells from settling during the fabrication process. A relatively quick gelation is subsequently required to retain the shape of the fabricated structure. This gelation is usually a cross-linking reaction initiated either by light, by a chemical, by hydrophobic or complexation interactions, or by a thermal transition. Both the shaping of the construct and this cross-linking reaction obviously should not compromise cell viability. Another requirement is adequate mechanical properties to retain the designed and fabricated shape. Most manufacturing processes impose stricter requirements on the mechanical properties of the gels than when casting and molding. Large structures with included porosity can only be accurately and reproducibly prepared when the elastic modulus and gel strength are sufficiently high.

Besides these constraints related to manufacturing, the hydrogel has to meet the demands for cell encapsulation and tissue development. Most hydrogels used in tissue engineering are chemically cross-linked, which means they are 3D networks of polymer chains with meshes that are orders of magnitude smaller than cells. This has a large restricting effect on the mobility of encapsulated cells; predominantly cell migration, as well as proliferation is completely arrested until degradation of the gel takes place [93]. However, degradation sites can be incorporated into hydrogels, allowing for cell-mediated matrix degradation permitting migration and proliferation [95-97]. Degradation of the matrix can also be hydrolytically driven [98], or even light-driven through incorporated photo-degradable

linkers [99]. Cell proliferation and migration are not always essential in the initial stage after encapsulation; in cartilage tissue engineering, for example, cells are often encapsulated at high densities with the aim of achieving high matrix production. Here, still the mesh size is important as it influences the diffusion of secreted proteins and glycosaminoglycans throughout the gel [100]. For the engineering of tissues where proliferation, remodeling and vascularization are required (Fig. 2), the hydrogel should allow space for these processes to occur. Designed macroporosity in the construct can aid in vascularization, as demonstrated by branched vascular networks becoming an integral part of a manufactured tissue [46].

A particular challenge in additive tissue manufacturing using cell-laden hydrogels is to develop a polymer along with processing conditions that are appropriate for both accurate printing and cell culture. Often, these criteria impose opposing requirements. For accurate printing of form-stable structures, high polymer concentrations and cross-link densities are desired, whereas for cell migration and proliferation and subsequent ECM formation both need to be low. For example, a currently used naturally-derived printable biopolymer, namely calcium-cross-linked alginate, has only a small processing window of in which both printing and cell culture are possible: the bioprinting window (Fig. 5A). This bioprinting window can be defined for other hydrogel systems by varying the polymer concentration and cross-link density and assessing the influence on printability and support for cell culture. Often the bioprinting window will be small, if at all present. The example in Fig. 5B shows a semi-quantitative assessment of the printability of alginate gels with a pressure-assisted microsyringe, in the form of a fidelity phase diagram. Two processing parameters are varied, the velocity of the micropositioners and the extrusion pressure, at two distinct hydrogel precursor viscosities (or concentrations), and the fidelity of the resulting structure is

assessed on a semi-quantitative scale ranging from a 'blob' structure to a high fidelity structure. The same group has also systematically investigated the effect of shear stress endured during the deposition on cell viability and function [101]. After several years of predominantly proof-of-principle studies demonstrating the (bio)printability of a gel with a particular AM system, researchers are increasingly optimizing gel parameters and processing conditions in systematic and quantitative ways.

Figure. 5.

Most attempts of additive tissue manufacturing so far have utilized hydrogels designed for purposes other than AM (Table 3). However, the development of polymers specifically for AM of cell-laden constructs has been explored to a limited extent, and may help overcome the limitations of current gels and expand the bioprinting window. One of the few examples of a hybrid gel tailor-made for AM is based on a PEG-PPO-PEG block copolymer. The thermosensitive block copolymer conveniently allows for dispensing a cell suspension at ambient temperature, which solidifies upon collecting at 37 °C. However, although most cells remain viable during the plotting process, the gel does not support cell viability in culture; all cells die within a few days, while the thermogel slowly dissolves into the culture media [42]. By functionalizing the terminal hydroxyl units of PEG-PPO-PEG with a peptide linker followed by a methacrylate group, a mechanism for covalent cross-linking, as well as biodegradability have been introduced, resulting in increased viability over 3 weeks of culture [104]. A similar approach of a synthetic gel that allows for both thermal gelation as well as UV-initiated chemical cross-linking was recently demonstrated by the same group [36]. The polymer is an ABA block copolymer composed of poly(N-(2-

hydroxypropyl)methacrylamide lactate) A-blocks and hydrophilic poly(ethylene glycol) B-blocks of a molecular weight of 10 kDa. The hydrophobic A-blocks not only induce lower critical solution temperature (LCST)-behavior employed for printing, but are also partly derivatized with methacrylate groups that allows for photo-polymerization for increased strength and shape stability.

Photo-cross-linkable gels that do not exhibit thermal gelation have also been printed. In one example, methacrylated dextran was mixed with high-molecular weight hyaluronic acid to obtain high viscosity for geometrical stability during printing [44]. Although the high viscosity enables printing of a porous structure that can be fixed subsequently by photo-cross-linking, the diameter and spacing of printed strands are considerably larger than for LCST-gels. It is expected that development of more hydrogels tailored for specific AM techniques will greatly increase the potential of AM.

Table 3

3.3.3. Scaffold-free tissue manufacture approaches

A relatively new trend in tissue manufacturing is the endeavor to use cells or aggregates of cells as building blocks to manufacture tissue engineering constructs without additional biomaterials. The rationale is that aggregates of cells can fuse through cell-cell and cell-ECM interactions to form larger structures, similar to embryonic development [106]. As cell-cell contact can be advantageous to direct tissue formation, it is believed that instead of suspended single cells, aggregates of thousands of cells (also referred to as tissue spheroids or embroid bodies) should be used for tissue manufacture. An elegant example of this approach (although still using agarose rods as a molding template) is the preparation of

vascular grafts from cell aggregates (Fig. 4D [46]). The beneficial effect of using high densities of cells and their associated ECM has also been demonstrated for cartilage repair [107]. Superficial and middle zone chondrocytes recovered after alginate culture were layered without additional biomaterials, resulting in continuous cell-derived tissues with different properties in each layer.

Another strategy that aims to engineer material-free implantable tissue is the so called “cell-sheet technology”. Cells are cultured on a thermo-responsive polymer-coated dish to form a self-supporting sheet of cells embedded in their self-produced ECM, which can be harvested by a reduction in temperature that renders the surface hydrophilic and hence cell-repellent [108]. In this way, cells can be harvested without destroying cell-cell contacts by trypsin. Over the last decade, cell-sheet technology has evolved to engineer several tissues with one or more cell types, and it has recently seen clinical applications [109]. So far, cell sheet technology has only been applied successfully for the regeneration of sheet-like tissues, such as the cornea, and as cardiomyocyte patches to repair partial heart infarcts. A next step in technological development is needed to create thick 3D tissue structures. Potentially, robotics could be employed to automate the cell sheet production process and to assemble 3D structures by stacking cell sheets, as the handling steps for cell sheet harvesting and stacking are fairly simple with high level of standardization. Obviously, many sheets are needed to build a substantial 3D tissue volume and the resulting high cell densities will require sufficient vascularization to sustain cell viability.

Recent technological development includes micro-patterned co-culture of fibroblasts and endothelial cells as a strategy to generate pre-vascularized tissue from stacks of cell sheets

[110]. Other potential approaches include the combination of cell sheets with dispensing techniques, to achieve a third dimension by deposition of structured hydrogels onto and in-between cell sheets. Either way, the current literature predicts that the cell sheet technology will play an increasingly important role in the additive tissue manufacture in the future.

4. Challenges and current developments

4.1. Construct design

A digital blueprint of an organ or tissue is a first requirement to produce an anatomically accurate TEC. Medical imaging techniques such as computed tomography and magnetic resonance imaging have been used to make anatomically shaped implants using intermediate moulds [111, 112] or by direct manufacturing [113]. More recently, 3D laser scanning was introduced to obtain digital 3D images of body contours, for example for the preparation of tailored breast prostheses implanted after mastectomy in breast cancer patients [114]. The obtained digital geometrical blueprint needs to be converted to a buildable, heterogeneous model representation describing material composition, distribution and geometrical information. Most AM techniques use only one material for building a construct, and only geometrical information is needed. Tissues however are heterogeneous, comprising of different ECM components, cell types and cell densities, such as the osteochondral tissue (Fig. 6). Methods have been developed to model and design functionally graded architectures with multiple biomaterials for AM [115-117]. These methods will need to be applied to approximate the complex nature of native heterogeneous tissues in manufactured cell-material constructs. Only in this way can one of

the major advantages of including cells in the fabrication process really be exploited, by deposition of different cell types according to the tissue blueprint.

Figure. 6.

The standard file format to feed geometrical information to AM control softwares is the STL format (Standard Tessellation Language). The format makes use of meshes of triangles that create watertight outer surfaces of objects. This works well for solid objects with limited complexity (which is usually the case for rapid prototyping of solid parts) that are to be built from a single material. Some AM control softwares give the user a degree of control over porosity, for example by controlling the filament distance that is used to create the tool path for deposition-based techniques. A novel modeling approach was recently introduced that automatically creates a tool path that fills set regions of a solid STL model, enabling to create distinct regions with variable porosity [121].

However, if the internal pore architecture is to be an integral part of the computer-aided design, the STL format an impractical one. An STL mesh of a few mm-sized scaffold with well-defined porosity easily exceeds one million triangles, taking up hundreds of megabytes of disk space and requiring heavy computation power to design and manipulate. However, the pore architecture of constructs with infinite volumes can be described using a single line of mathematical equation, with freedom to design different pore shapes, pore sizes and porosity, and allowing to include features such as porosity and pore size gradients [69]. A more versatile file format that would allow combining such a porosity function with a mesh that describes the macroscopic shape of an organ would make designing and manufacturing

tissue and organ constructs much more achievable. Until then, computer designs of porous structures will be restricted to either a coarse porosity for large models, or small structures in the case of finer, well-defined porosity.

A new route to create porous models from medical imaging-derived data was recently developed [122]. Existing methods were adapted that convert CT-derived anatomical data into a volumetric mesh that can be used *e.g.* for studying biomechanics using finite element modeling [123]. In this case, the mesh is used to create a completely interconnected strut-based porous model. In practice, the solid model obtained by imaging is seeded with points at a given distance (seeding distance SD), which are connected by the finite element software to result in a 3D mesh of tetrahedrons. Subsequently, struts of a given thickness (ST) are designed around each edge of all tetrahedrons, and these struts are joined at their intersections to create a watertight model. Using this method, one can generate porous models that have the overall shape of the scanned tissue and/or organ, built up from fully connected straight struts to ensure manufacturability and optimal mechanical stability. The pore size and porosity can be tailored by controlling the density of seeding points in the creation of the tetrahedron mesh, as well as by choosing an appropriate strut thickness (Fig. 7). The example given in this review demonstrates how from a solid breast model obtained by 3D scanning, a range of scaffold morphologies and porosities can be designed and fabricated to the requirements of the project objectives and aims.

Figure 7

4.2. Hardware

Initially, tissue manufacture has focused on the use of inkjet and laser printers. However, printing is inherently a 2D process. Inkjet printers are not designed to fabricate 3D structures. The upper threshold for viscosity of the ink (30 mPa·s) excludes the use of many hydrogels and impedes the build-up of large 3D structures. This limitation also applies to biolaserprinting in its current form, and to laser direct writing. To construct functional tissues and (ultimately) organs, techniques are required that are capable of building structures at relevant scales and accuracies. We conclude that AM techniques possess this capability.

Existing AM devices are currently being modified to facilitate tissue manufacturing. This often entails control of the environmental properties (temperature, humidity, and sterility) and downscaling of containers, feeders, etc., to reduce loss of costly biomaterials and cells. Over the last few years, AM devices designed particularly for tissue manufacture have become commercially available, with an emphasis on robotic dispensing techniques [124]. Dispensing is a technologically straightforward method to create designed structures at relatively high speeds. The largest challenge for the dispensing technology component is to build tissues with high accuracy. Liquid precursors need to be dispensed in thin strands from small-diameter tips and solidify quickly before spreading out initially on the platform and later on the subsequent layer (a video of dispensing hydrogels is available online as Supplementary Information). When only materials are dispensed, this can be achieved by employing high polymer concentrations and a non-solvent for quick coagulation. In this way, well-defined structures have been prepared from filaments of only 1 μm diameter [71]. However, for encapsulating cells non-solvents cannot be used and polymer concentrations must be lower, so cell-laden hydrogel structures typically have strands with diameters of 100 μm or larger.

Light-based curing techniques are generally more accurate than dispensing techniques. With photo-lithography and micromolding, cell-laden microgels with well-defined geometry of up to several hundreds of μm have been prepared [125, 126]. Two-photon polymerization (2PP) has been employed to locally functionalize hydrogels with RGD-peptide sequences, leading to directed cell migration with accuracy below 100 μm. As a light-directed AM technique, stereolithography can be performed at a large scale-range; from decimeter-sized objects down to sub-micron features can be built. Such high accuracy, combined with high versatility and freedom of design (particularly compared to dispensing techniques) results in the ability to create highly detailed organic shapes, such as the alveoli (Fig. 8.), fabricated by 2PP-based microstereolithography [127]. The woodpile structure in the bottom row of Fig. 8 would not be functional as a scaffold for the pores are too small to facilitate cell ingrowth, but does illustrate the high level of geometric control that can be achieved with optical techniques. Well-defined structures have been prepared at a resolution of several tens of μm from hydrogels that were also used for cell encapsulation using the same stereolithography setup, although complex and clinically relevant sized hydrogel structures with encapsulated cells at such resolutions still await to be reported.

Figure. 8.

In the authors' opinion, the largest challenges to overcome for light-based techniques are long fabrication times, and gravitational settling of cells in the precursor solution. One of the first reports on stereolithographic fabrication of hydrogel structures in 2005 argued that the stereolithography fabrication process was too slow for cell encapsulation; however,

controlled spatial distribution of cell-adhesive peptides could lead to controlled cell seeding and diffusivity throughout the scaffold, which in addition to the presence of channels would be superior to traditional seeding and culturing cells on scaffolds [128]. More recently, a modification of a stereolithography apparatus for the fabrication of PEG-diacrylate-based hydrogel structures with encapsulated cells was reported (Fig. 9) [70]. To prevent cells settling to the bottom of the tank due to gravity, each layer of cell-containing prepolymer solution was manually added prior to curing of that layer. Besides achieving a homogeneous cell distribution, this also allows to use multiple gel compositions and cell types, which is not generally possible using the stereolithography technique [68]. In this case the cell suspension is still dispensed manually, but one can easily envision automation of this step.

Figure. 9.

Another approach for partially automated layered photo-patterning of cell-laden hydrogels uses masks printed on a commercial high-resolution printer [129]. A UV curing unit was employed with the masks to cure a PEG-DA cell suspension in a chamber that was replaced for each subsequent layer, with washes and refilling in-between. The researchers performed an extensive biological characterization including optimization of the gel system (among which type and concentration of adhesive peptides) and demonstration of the increased metabolic activity of hepatocytes encapsulated in perfused patterned hydrogels as compared to bulk hydrogels.

Besides layer-by-layer deposition just prior to photo-cross-linking, other solution paths to cell settling are possible. For example, by continuous tumbling of the setup, gravity can be

counteracted by centrifugational forces, creating a micro-gravity environment in which cells do not settle. Otherwise, a physical gel could be employed as the (chemical) hydrogel precursor in which cells do not settle, rather than using a liquid solution. In this case, a 3D pattern could be cross-linked by moving the focal volume of one laser, several lasers creating an interference pattern, or by 2PP. After cross-linking of the 3D structure, the non-cross-linked volume including cells could be removed by reversing the physical gelation (for example, warming up of gelatin-methacrylate or ion exchange for an alginate-based gel) and recovered for later use, leaving a porous and structured hydrogel with encapsulated cells.

Even if settling of cells is prevented, speed still is an important processing parameter. When working at higher resolution it generally takes longer to build up a specific volume, and this is also the case for stereolithography. However, new technologies are being developed to increase production speed. For example, as opposed to illumination by a computer-controlled laser tip drawing over the surface in most conventional SLAs, some new apparatus are equipped with a digital mirror device that enables projection of a whole layer at once, thereby significantly increasing fabrication speed [35, 68].

A current development in stereolithographic AM that aims at high-throughput manufacturing of accurate multi-material parts by a new process named stereo-thermal-lithography [130]. It employs UV radiation and thermal energy (produced by IR radiation) simultaneously to initiate the cross-linking polymerization reaction in a medium containing both photo- and thermal initiators. The amount of each initiator is low enough not to start polymerization by only one of these two effects. However, at a point where the two effects coincide, the amount of radicals generated is sufficiently high to initiate the polymerization process. Temperature is used to both produce radicals through the fragmentation of

thermal initiators and simultaneously to increase the initiation and reaction rate of the photo-initiated curing reaction. Added to this system is a rotating multi-vat that enables the fabrication of multi-material structures (Fig. 10).

Figure. 10.

4.3. Biomaterials

For application in additive tissue manufacture, biomaterials must meet more stringent requirements than for most other applications such as in food, pharmaceutics or sensors. Nevertheless, some innovations from other fields might possibly be translated to AM techniques and cell encapsulation, using alternative components and processing conditions. This section gives an overview of such developments.

4.3.1. Degradation properties

Polymer network chains give hydrogels their mechanical stability, but at the same time restrict the mobility for cells to migrate and proliferate. Therefore, it is important to match the kinetics of degradation with firstly the cell migration and proliferation and subsequently tissue formation, such that the newly deposited ECM can take over the load to a certain extent from the partially degraded polymer network. Moreover, the rate of tissue formation and remodeling depends on many factors and is different for various tissues. Hence, it is of utmost importance to study those *in vitro* and/or *in vivo* mimetics in great detail [4].

By far most developments on degradable hydrogels for cell encapsulation have been based on the water-soluble, bioinert polymer poly(ethylene glycol) (PEG) [93]. In itself it is a non-

degradable polymer, but PEG oligomers that are low enough in molecular weight to be secreted by the kidneys are often the basis for the synthesis of degradable macromers [131]. These can be (photo)polymerized from aqueous solutions with suspended cells, to form cell-laden hydrogels [132]. The degradation kinetics of these gels can be tuned by variation of the polymer concentration and molecular weight, the choice of degradable co-monomer and the ratio of PEG to the degradable component. Furthermore, different cross-linking mechanisms lead to different network structures with varying degradation profiles (Fig. 11). Addition type chain-cross-linking, step-growth end-linking and mixed-mode mechanisms all allow cell encapsulation and the inclusion of biologically functional entities such as cell-adhesive peptides or tethered growth factors [133], but differ in other respects. The chain cross-linking mechanism is particularly attractive for AM techniques because of the fast reaction and spatially directed initiation by light such as in stereolithography. However, end-linking polymerization reactions are characterized by a particularly large control over the network architecture. For example, it has enabled the preparation of gels with the peptide link GCRD-GPQG↓IWGQ-DRCG, which is cleavable at the "↓" site by cell-secreted matrix metalloproteinases (MMPs) [96]. These MMP-cleavable gels (also supplemented with covalently bound cell-adhesive peptides) showed ingression of migrating and proliferating fibroblasts seeded on the surface of the gels, which was not observed in the control gel that was cross-linked with an MMP-insensitive peptide linker. Later studies have also shown the possible application of such gels for cell encapsulation [134]. In this way, cells can proliferate, migrate and form new tissue while parts of the gel that are (still) free of cells remain untouched, retaining the overall shape and mechanical stability of the gel. This strategy of cell-mediated degradation mitigates the challenge of

tuning hydrolytic degradation with tissue formation, which is a very site- and condition-specific process and therefore difficult to predict.

So far, fabrication of cell-laden PEG structures by AM techniques have mostly employed off-the-shelf PEG-diacrylates in conjunction with RGD-PEG-acrylate, resulting in gels that support cell viability but are non-degradable and therefore of limited use in tissue engineering. However, it can be easily envisioned that the aforementioned strategies of introducing hydrolysable links, tethered grow factors and enzyme-sensitive cleavage sites will also be used with alternative hydrogel platforms such as thermo-sensitive PEG-PPO-PEG, allowing the application in AM technologies.

Figure. 11.

4.3.2. Mechanical properties

A specific disadvantage of hydrogels is that their mechanical strength is mostly far below that of load-bearing tissues, such as cartilage. This fact has not been appropriately addressed by researchers working with hydrogels in tissue engineering, particularly for cell encapsulation. The lack of strong and tough hydrogels is one of the main limiting factors in advancing tissue manufacture to larger scales and better quality of TECs.

Hydrogels are intrinsically weak due to the high content of water, which dilutes the network of elastically active chains and reduces physical entanglement. Obvious ways to increase a gel's modulus and strength are increasing the polymer concentration and cross-link density; however this is often detrimental for cell viability and function [95], and for the production

and distribution of matrix components [100]. Recent developments in areas outside of biomedical engineering have resulted in hydrogels with novel chemical structures that have considerably improved mechanical properties due to the introduction of an energy-dissipating mechanism, thereby increasing toughness and (tear) strength while still containing high water volume fractions [135]. These include slide-ring gels, double-network gels and nanocomposite gels. Particularly the latter two classes of gels show very high toughness and compressive strengths, while retaining flexibility and high water-content. Gong *et al*. have prepared double-network gels with remarkably high compressive strengths of up to 17 MPa at a similarly remarkable strain at failure of 92 %, while the water content was as high as 90 % [136].

Relatively densely cross-linked networks were swollen in solutions of a second monomer and cross-linker, followed by formation of the reinforcing second, interpenetrating network, which has a relatively low cross-link density but higher concentration than the first network. As a result of the high degree of swelling in the monomer solution, the first gel network is highly extended in the final product while the second network is relaxed, which results in much stronger reinforcing effects than in conventional interpenetrating networks. These networks are currently under investigation for use as artificial articular cartilage with promising initial results [137]. In the translation of the double-network strategy to cell encapsulation and tissue manufacturing, the major problem is that low-molecular-weight monomers are generally cytotoxic [138]. However, reinforcement of gels with encapsulated cells using this strategy is more feasible using double bond-functionalized macro-monomers of intermediate molecular weights, or with other non-cytotoxic network-forming components such as physically cross-linked gels or self-assembling peptides [139].

Nanocomposite gels are another class of hydrogels exhibiting mechanical properties superior to conventional hydrogels. These are water-swollen networks of hydrophilic polymers, physically cross-linked through adsorption of the polymer chain ends on nanometer-sized inorganic (clay) platelets. The nature of the cross-links being considerably large planar sheets with high junction functionality somehow yields unusual mechanical properties, including very good toughness, high elongation at break and ultimate stress (both in tension and compression) up to several MPa [140]. Generally, they are synthesized through the *in situ* free-radical polymerization of N-isopropylacrylamide (NIPAAm) in an aqueous mixture with clay particles. Recent developments included the replacement of the redox initiating system with photo-initiation, greatly increasing the usefulness for patterning and processing with AM techniques to form designed structures. Poly(NIPAAm) nanocomposite gels can be used for cell culture on the gel surface and subsequent detachment of cell sheets without the use of proteases (see also section 3.3.2) [141], but the *in situ* polymerization of the toxic monomer NIPAAm does not allow for cell encapsulation. However, most recently nanocomposite gels have successfully been prepared from four-armed PEG macromonomers of 20 kg/mol molecular weight [142], which is a big step towards the applicability of this gel reinforcing strategy in additive tissue manufacture.

Besides double-networks and nanocomposite gels, other approaches have been followed attempting to synthesize strong hydrogels for a variety of applications, some of which may be translatable to tissue manufacture. The interested reader is referred to the review by Calvert [143] for a more comprehensive overview.

4.3.3. Hybrid structures

Since water is a crucial component of living systems and a major component in most tissues, the processing of cells into designed hydrogel structures seems a logical approach. However, most organisms are not only composed of hydrated cell-rich tissues, but also of more 'dry' and protein-rich ECM such as bone and tendon. Therefore, co-manufacturing of solid biodegradable material (polymers, ceramics) with cell-laden hydrogels could combine favorable mechanical properties with cells positioned at defined locations at high densities. Recently, this approach was successfully applied for the generation of organized viable constructs by alternate deposition of thermoplastic fibers and cell-laden hydrogels (Fig. 12) [144]. The resulting mechanical properties of the constructs were significantly improved and could be tailored within the same range as those of native tissues. Moreover, the approach allows the use of multiple hydrogels, and can thus build constructs containing multiple cell types or bioactive factors. Furthermore, since the hydrogel is supported by the thermoplastic material, a broader range of hydrogel types, concentrations and cross-link densities can be used compared to the deposition of hydrogels alone, thereby improving the conditions for encapsulated cells to proliferate and deposit new matrix.

Figure. 12.

A different possible approach is by taking advantage of the membrane-forming self-assembly process that occurs when solutions of hyaluronic acid and particular types of peptide amphiphiles are brought into contact [145]. Computer-controlled deposition of one of the components with suspended cells into the second component would lead to a cell

suspension (potentially gelled by photo-cross-linking) immobilized in membrane-surrounded strands.

4.4. Vascularization

In spite of considerable attempts in bioengineering functional tissues and organs, most applications of tissue engineering have been restricted to avascular or thin tissues, as without blood vessels, nutrients and oxygen cannot diffuse into and out of TECs to retain cellular viability. As cells existing more than a few hundred microns away from the nearest capillaries would undergo hypoxia, apoptosis and ultimately cell death, vascularization is one of the major challenges tissue engineers are faced with in the 21st century. Particularly with the demand from a clinical point of view to fabricate large TECs in which overcoming transport limitations becomes increasingly difficult. From a tissue transplant point of view, it is well know that grafts can spontaneously vascularize after implantation, due to an inflammatory wound-healing response and the hypoxia-induced endogenous release of angiogenic growth factors [151]. The process of angiogenesis follows from a complex cascade of events including ECs activation, migration, and proliferation, their arrangement into immature vessels, addition of mural cells (pericytes and SMCs), and matrix deposition as the vessels mature [146]. The molecular mechanisms regulating each of these stages are being described, and it is obvious that different growth factors act at distinct steps of neovascularization. Nevertheless, this induced vessel ingrowth is often too slow to provide sufficient nutrients to the cells in the center of the transplanted tissue. Conclusively, the limiting step is therapeutic angiogenesis, and both microvascularization and macrovascularization are required to provide nutrients and oxygen in 3D.

Vascularization with or without biochemical stimulation (e.g., growth factor delivery), either through ingrowth from surrounding tissues or through *de novo* blood vessel formation from co-deposited cells is currently investigated by a number of research groups [147-150]. Mimicking biological patterning may be especially useful to control tissue development processes such as neovascularization, where unguided or uncontrolled growth can lead to pathological effects including tumor growth, metastasis, and deformed vessels. Techniques developed for microarray patterning, microcontact printing, micromolding and laser photolithography can be translated to AM of tissues to form gradients of growth factors within the scaffolds or to co-deposit cells. These are highly architecture-dependent processes that can benefit from the specific advantages of AM techniques.

Several strategies for vascularization at different levels are being developed [151], as illustrated in Figure 13. First of all, the micro-architecture of any scaffold must allow blood vessel ingrowth, thus a pore network with large enough interconnections is a prerequisite. AM-produced scaffolds generally have better interconnectivity and lower tortuosity than scaffolds fabricated by conventional techniques such as porogen leaching. In addition, different levels of porosity can be designed to allow cells to fill smaller pores with new tissue while leaving large pore channels available for vascularization (Figure 13A). Such scaffold designs would also be beneficial in combination with *in vivo* prevascularization strategies; the use of tortuous scaffolds fabricated by thermally-induced phase separation or particulate leaching with arteriovenous loops *in vivo* has lead to the formation of vascularized tissue but in pores with small interconnections too distant from the AV loop the tissue was prone to necrosis in the longer implantation time points [153] (Figure 13B). Furthermore, AM techniques can aid vascularization by site-specific delivery of angiogenic factors, possibly released on demand by cell-produced enzymes (Figure 13C). Finally, AM

can be employed for the precise co-deposition of gels with relevant cells types (endothelial cells, myoblasts, fibroblasts) to guiding migration, proliferation and network formation. This will likely improve and accelerate *in vitro* prevascularization (Figure 13D).

Figure 13.

4.5. Scale-up of the AM process

An additional challenge in the development of current lab-based attempts of tissue manufacture towards clinical application is the gradual scale-up of the process. Going from the millimeter scale that current work is focused on, to the centimeter scale of tissues or – eventually– the decimeter scale of organs implies an increase in material volume, numbers of cells and possibly construction time by a factor of 10^3 or 10^6, respectively. With such scale-up, transport limitations, as well as acquiring adequate cell quantities become increasingly difficult. Currently, to obtain sufficient numbers of cells cell populations (either differentiated cells harvested from a patient or stem cells) are mostly expanded in 2D monolayer using tissue culture flasks. The manual seeding, splitting and harvesting is not only labor-intensive and expensive but also lacks high reproducibility, and most importantly it is also insufficient for obtaining large enough numbers of cells for manufacture of TECs of clinical relevance. Additive tissue manufacture techniques might not develop without the concurrent development of automated 3D cell culture systems [23], which can be based on suspended microcarriers [156] or fluidized bed bioreactors [157]. This concept however appears slow to be embraced, and a roadmap has been established to overcome scientific, regulatory and commercial challenges in order to implement a new bioreactor-based paradigm [158].

Furthermore, by seeding of pre-fabricated scaffolds with cells, the seeding density and efficiency that can be achieved may not be as high as by encapsulation of cells inside hydrogels. Native tissues often contain millions of cells per mL of volume, and the direct manufacture of tissue precursors with similar cell densities might be a better approach than the preparation of constructs with considerably lower densities, requiring extensive *in vitro* culture. This approach has been followed by the preparation of cell-laden hydrogels by photo-initiated cross-linking of methacrylated gelatin and hyaluronic acid with densities up to 100 million cells/mL [91].

4.5 Regulatory and commercialization aspects

Fundamentally, academia and business operate on very different models. Academia has the need to publish results first, and emphasis is put on the ability to first discover a method or technique; there is often little, if any, reward for a researcher who perfects the technology or verifies and expands the initial results, even if the modified process is a substantial leap over the original research. However, in today's world time is a precarious factor and hence manufacturing R&D must closely follow discovery to ensure that companies can transform innovation into products invention and business performance in the tissue engineering industry [159]. As a result, the speed at which small enterprises and the biotechnology industry at large can translate AM research into high-value-added products and high efficiency processes is critical. Realizing this potential requires progress on many fronts of science and engineering. Government funds for regenerative medicine research have created some of the most sophisticated institutes and laboratories around the world. Yet, research to date has been largely focused on the discoveries with a notable absence of

capabilities and funds focused on scale-up of manufacturing or clinical trials, which would allow moving the research from bench to bedside [15, 160, 161].

Scale-up of manufacturing processes from small lot sizes to mass production poses the first key challenge for the fabrication of TECs. Biomaterial scientists in close collaboration with engineers need to upscale current lab-based technologies to economies of scale that allow reduced manufacturing costs and accelerated entry of TECs into commercial applications. Integrating bottom-up and top-down processes into new manufacturing paradigms is the second key challenge. Today's first and second generation scaffolds are frequently manufactured with traditional biomaterials and/or manufacturing techniques, which can be prohibitively expensive and/or have limited throughput to reach economics of scale [8].

As with all tissue engineering and regenerative medicine products (particularly cell-based ones), TECs fabricated by AM will have to go through a long and costly trajectory of toxicity testing, pre-clinical testing and clinical testing. Analogous to drug master files, material master files will have to be obtained for each material. For some materials that have been used with AM such as PCL and PLGA, these material master files have been obtained on behalf of companies manufacturing those polymers. New devices based on these (non-modified) materials can get clearance by the Food and Drug Administration (FDA) relatively fast and easily through the so-called 510(k) process [161].

Growing barriers between clinical and basic research, along with the ever-increasing complexities involved in conducting clinical research, are making it more difficult to translate scaffold-based tissue engineering concepts to the bedside [162, 163]. The challenge is therefore to manage the broad spectrum of stakeholder expectations compounded by the sea of ambiguity that swirls around the evolving regenerative medicine industry including its yet to be established supporting business models. Quite correctly

patients want therapies today, investors need returns quickly and regulators require safety and efficacy studies of adequate length to reassure themselves of the worthiness of the medical product before it can be approved for routine clinical use. A great nonmedical technology based idea, such as the iPAD, can be invented today and commercialized within weeks to months. However, as the medical sector is all too aware, the same is not true for medical devices, and certainly not for regenerative medicine-based therapies. These challenges are limiting commercial interest in the field and hampering the clinical research enterprise at a time when it should be expanding to 'translate' fundamental research results into practical applications [164]. The translational pathways for clinical testing and therapeutic use and the complexity of TECs, often containing a combination of scaffolds, cells, and/or growth factors, creates challenges for product characterisation, regulatory approval and manufacturing conforming with GMP. Hence, it is necessary to develop a road map with low and permeable barriers and a great deal of interaction between academic research and industry practice that then eventually provides resources and endorsements to help product developers to improve the safety and effectiveness of engineered tissues ready for testing in clinical trials.

Successful commercialization ultimately requires regulatory and reimbursement approval, and in regard to the former, although the FDA is making progress in the regulation of scaffold/cell-based therapies, a thoroughly revised system is needed for the regenerative medicine products of the 21st Century. Regulatory agencies thus must develop and approve in due time the necessary and appropriate processes for regulating the delivery of safe and effective clinical therapies based on advances in regenerative medicine [165].

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enterprise at a time when it should be expanding to 'translate' fundamental research results into practical applications [164]. The translational pathways for clinical testing and therapeutic use and the complexity of TECs, often containing a combination of scaffolds, cells, and/or growth factors, creates challenges for product characterisation, regulatory approval and manufacturing conforming with GMP. Hence, it is of necessity to develop a road map with low and permeable barriers and a great deal of interaction between academic research and industry practice that then eventually provides resources and endorsements to help product developers to improve the safety and effectiveness of engineered tissues ready for testing in clinical trials.

Successful commercialisation ultimately requires regulatory and reimbursement approval, and in regard to the former, although the FDA is making progress in the regulation of scaffold/cell-based therapies, a thoroughly revised system is needed for the regenerative medicine products of the twenty-first century. Regulatory agencies thus must develop and approve in due time the necessary and appropriate processes for regulating the delivery of safe and effective clinical therapies based on advances in regenerative medicine [165].

5. Future directions

Just as advances in information technology, materials, imaging, nanotechnology and related fields — coupled with advances in computing, modeling and simulation — have transformed the physical sciences, so are they beginning to transform life science [166]. Most recently the term convergence has been introduced to describe this change process also in the biomedical field. In general terms convergence was defined and it has in large stimulated our rethinking of how scientific research can be conducted. A major outcome of this

rethinking is that areas such as additive tissue manufacturing not only require collaboration among research groups but, more deeply, the integration of disciplinary approaches that were originally viewed as separate and distinct. This merging of technologies, processes and devices into a unified whole will create new pathways and opportunities for scientific and technological advancement in the targeted field. Based on this background, we will describe in this section our thinking process related to aspects expected to play an important role in the future development of the AM techniques.

5.1. Modular tissue assembly

As upscaling and automation are specific advantages of AM techniques and a major driving force for developing these techniques for tissue assembly, the associated complexities will have to be addressed. One approach to solve issues of accuracy, reproducibility, and error scaling is to implement the concept of so called “digital fabrication”. It refers to actual printing of physical building blocks termed voxels, as opposed to analogue (continuous) material commonly used in most conventional manufacturing techniques [167]. The voxels are characterized by their self-aligning and interlocking properties, which enable one to fabricate objects that are more precise than the fabricator that created it. This is analogous to a child with 1 mm hand placement precision assembling LEGO structures with 5 μm precision. Furthermore, while with analogue techniques errors in accuracy accumulate when structures are scaled up, in digital fabrication errors tend to average out.

Several groups using cell aggregates with or without cells have followed the modular approach of using standardized building blocks to build up larger structures. For example, cell aggregates or microtissues can be fabricated in pre-designed shapes by seeding and

culturing in micro-molded well plates and serve as building blocks to assembly multi-cellular tissues at a higher level of organization [168]. Otherwise, micro-engineered cell-laden hydrogels can be shaped into larger tubes, sphere shells and other shapes gel blocks by self-assembly when the gels are being restricted at a surface and forced to form a close packing [169]. Such strategies might evolve into AM technologies on their own, or it might become possible that cell-laden gel blocks such as these be fed to a voxel printer to actively assemble complex tissue constructs. Such an approach could also address the issue of vascularization, by assembling microgels pre-seeded with endothelial cells and perfusion of the interstitial space with medium in a bioreactor [170].

5.2. Convergence of techniques

All AM techniques have their specific shortcomings and advantages, particularly in combination with specific biomaterials. So far, mechanical engineers have been working with widely available polymers in their attempts to develop new manufacturing techniques, and biomaterial scientists have been working with commercially available devices in their attempts to develop new biomaterials. We foresee the convergence of skills and techniques to take AM to a higher level.

The example of stereolithography combined with stepwise addition of material discussed in section 4.2 can be further developed into a combined robotic dispensing and stereolithography system, in which a layer with coarse structures is first deposited, followed by more accurate local modification by a computer-controlled laser. These modifications can involve cross-linking of the hydrogel precursor with the intention to discard non-cured material afterwards, but it can also be localized matrix modifications such as the increasing

of the cross-link density to obtain anisotropic properties, or functionalization of the gel with cell-adhesive peptides [171]. Another method to modify the direct cellular environment that has not yet been explored is to initiate modification reactions using the cells as intermediate. For example, fluorescent light emitted by fluorescein-tagged cells could set off reactions in the direct cell surroundings by using eosin Y as an initiator. Fluorescein can be excited by an argon laser at 488 nm (blue) and emits around 520 nm (green), which is the wavelength around which eosin has a narrow absorption band. Therefore, it would only be excited by the green light emitted from cells rather than by the incident blue laser light and could as such initiate local reactions. This photo-initiator has been previously employed successfully for cell encapsulation [172].

Other post-manufacture modifications with different techniques to achieve features at higher resolutions could include drilling or laser ablation of channels in gel blocks. Such channels could be used for perfusion of medium during culture, or for neo-vascularization by seeding an (co-)culture of endothelial cells and fibroblasts on the inside walls of such channels.

5.3. Automation of pre- and post-manufacturing phases

While the successful translation of cell-based therapies from bench to bedside has, at least in part, been complicated due to regulatory issues, the automation of the production phases could facilitate the progress of tissue engineering towards clinical application. Therefore, it is also important that the assembly phase is integrated with other stages in production and culture of tissue constructs with reduced manual intervention. Besides automation of the pre-manufacture cell-culturing phase, also post-manufacture cultivation of tissue constructs

requires automation and integration with the fabrication phase. 3D hydrogel-based constructs with embedded cells can, for example, be produced in one zone of an isolated system by means of a multiple-head bioprinter, while afterwards the 3D construct can be transferred by a precision robotic arm to a subsequent zone for culture within a bioreactor under controlled dynamic conditions. Environmental (aseptic) conditions, including humidity, temperature and CO_2 can be controlled and monitored within such systems, and the integration of the different production stages into a single device will significantly reduce the risks of contamination, increase the productivity and will thus increase the reproducibility facilitating the ultimate compliance with regulations [158, 173].

5.4. Manufacturing of tissue-like constructs for drug discovery and/or testing

Tissue manufacture has predominantly been discussed from a regenerative medicine perspective. However, the manufacture of tissue-like constructs can also be of benefit to the fields of drug discovery and testing, and for studying disease processes and developmental biology. Such studies are being performed using 2D patterning techniques, to quickly test the interaction of many parameters via high-throughput screening [174].

A 3D environment however could provide a more appropriate model than 2D environments, which might make drug screening more selective and disease process studies more relevant. Printed microtissues will provide a valuable step in the development process of drugs, by yielding extra information before expensive and complex *in vivo* trials. This concepts lead to the automated fabrication of tissue-like living constructs not only for regenerative medicine, but also as *ex vivo* drug screening models [175] or for cancer research [24]. It remains to be seen if AM techniques will reach similar accuracy and level of complexity as 2D lithography, but there will undoubtedly be cases in which the added value of a 3D environment

outweighs the limited spatial resolution. A particular advantage of these areas of application is that the required scale of manufactured tissues is much smaller than for regenerative medicine, so these AM techniques can be used for such applications before the intended scale-up to organ-sized constructs with integrated vascular networks has been realized [176]. In addition, such concepts can be translated comparatively fast into real world applications as they do not need to undergo the regulatory route of implants.

5.5. *In situ* additive manufacturing

Apart from fabrication of pre-designed constructs, AM techniques are currently being developed for *in situ* fabrication [177, 178]. This entails the deposition of material into an *a priori* unknown recipient site, requiring an adaptive system that is capable of performing real-time imaging, registration and path planning. Cohen *et al*. printed alginate into complex osteochondral defects in a calf femur model [177]. The alginate cross-linking was initiated prior to the printing process by mixing in divalent ions, such that no post-processing steps were required. Although still in its infancy and presenting considerable technological challenges, *in situ* AM appears to have great potential for clinical applications that require a minimally invasive and/or geometrically patient-specific treatment concept. The adaptive nature of the process makes it a particularly attractive, omitting the necessity for imaging, pre-designing and implantation of a pre-fabricated construct. *In situ* AM could potentially be extended to many fields of trauma surgery and much is to be expected from these developments.

6. Conclusion

In summary, additive manufacturing will enable the production of cell-containing constructs in a computer-controlled manner, thereby bypassing costly and poorly controlled manual cell seeding. Although big steps have been taken since the origins early in the past decade, the technology is still in its infancy. It is now critical to address key issues in biomaterials development (matching degradation to cellular production and providing adequate mechanical properties, while achieving rheological properties required for the manufacturing process), construct design (including vascularization of the construct), and system integration (inclusion of multiple cells, materials and manufacturing processes in a sterile and controlled environment). It is also important to pursue the development and commercialization of constructs in a manner that is acceptable to regulatory agencies, such as the Food and Drug Administration, where they will more than likely be classed as "combination products", to efficiently translate research outcomes to clinical benefits. With the joint effort of researchers combining chemistry, mechanical engineering, information technology and cell biology, AM techniques can evolve into a technology platform that allows users to create tissue-engineered constructs with economics of scale in the years to come.

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Fig. 1. Example of cell-free clinical application of tissue engineering: calvarial reconstruction using polycaprolactone-calcium phosphate scaffolds. A. Scaffold designed from medical CT imaging data and fabricated by fused deposition modeling B. Calvarial defect C. Defect after implantation of scaffold D. CT images showing beginning bony consolidation of the defect after 6 months. Reproduced with permission from (2011) Georg Thieme Verlag KG [11].

Fig. 2. Schematic elucidating the principle of additive tissue manufacturing. A. Imaging of an organ to obtain 3D digital blueprint. B. Concurrent additive manufacturing of scaffolding structure (biodegradable thermoplastic) and cells suspended in gels: pre-adipocytes in adipose-mimetic ECM gel and smooth muscle cells in gel mimicking their native ECM. C. Manufactured 3D neo-tissue construct. D. Implantation after mastectomy.

Fig. 3. History of additive manufacturing and its application in tissue engineering; the introduction of technologies and major scientific findings.

Fig. 4. Examples of bioprinted structures. A, B, C: layer-by-layer fabrication of gelatin/alginate/fibrinogen containing adipose-derived stem cells (in pink) and hepatocytes in gelatin/alginate/chitosan (white). D: fusion of printed cell aggregates for scaffold-free vascular tissue engineering. E: hepatocytes in gelatin/chitosan hydrogel structures 1 month post-dispensing. Reproduced with permission from (2009) Elsevier [46] and [40, 75] copyright © 2005, 2009. Reprinted by Permission of SAGE.

Fig. 5. A. Processing window for bioprinting of alginate hydrogels cross-linked by divalent calcium ions. Printing imposes minimum values for alginate and calcium concentrations. Cell

culture imposes maximum values, leaving a small window for bioprinting. B. 3D phase diagram of microfabrication of sodium alginate/calcium at two distinct alginate concentrations, showing the influence of velocity of the micropositioners and extrusion pressure on the fidelity of the final shape. Reproduced with permission from (2009) ASME [102] and (2009) IOP [103](doi:10.1088/1758-5082/1/4/045002).

Fig. 6. Example of functional graded construct design for osteochondral tissue. The differences in tissue composition, mechanical properties and cell type in the native tissue are reflected in the design for the manufacturing process by material/hydrogel composition, construct architecture and encapsulated cell type. Reproduced with permission from (2009) Future Medicine [118] (2009) Mary Ann Liebert [119] and (2009) Wiley [121].

Fig. 7. Generating personalized scaffolds for breast reconstruction. Top-row: CAD-data of solid model and porous 'skeleton-mesh'. Middle row: CAD models with varying pore size and porosity as a result of different seeding distances (SD) and strut thicknesses (ST). Bottom row: physical prototype models manufactured by fused deposition modeling. Reproduced with permission from (2011) IOP [103](doi:10.1088/1758-5082/3/3/034114).

Fig. 8. Two examples of structures prepared by two-photon polymerization (2PP) techniques. A. CAD image of a pulmonary alveolar fragment. B. SEM image of a fabricated alveolus. C, D. Woodpile structure resembling an FDM-fabricated scaffold, albeit at about 100x smaller scale. Reproduced with permission from (2011) Springer.

Fig. 9. A schematic representation of the 'bottom-up' SLA modification, in which the prepolymer solution is pipetted into the container one layer at a time from the bottom to the top. [70] - Reproduced by permission of The Royal Society of Chemistry.

Fig. 10. The stereo-thermal-lithographic process with multi-vat system. Liquid resins are solidified locally by co-illumination from a UV (light) and an IR (heat radiation) source, both patterned using computer-controlled digital mirror devices. The rotating multi-vat system enables the construction of multi-material constructs Reproduced with permission from (2011) Springer [130].

Fig. 11. Schematic representation of the initial monomer molecules and cross-linked polymer networks formed through (A) chain-cross-linking polymerization mechanism (B) end-linking mechanism and (C) mixed-mode mechanism.

Fig. 12. Co-manufacturing of solid biodegradable materials with cell-laden hydrogels. A. Schematic overview of a hybrid bioprinting process encompassing alternating steps of printing polymer and cell-laden hydrogel, yielding hybrid constructs. B. Layering of the dye-containing alginate results in specific confinement of the printed hydrogels C. High cell viability as demonstrated by fluorescent Live/Dead assay. Scale bars represent 2 mm. Reproduced with permission from (2011) IOP [144] (doi:10.1088/1758-5082/3/2/021001).

Fig 13. The most direct approach to providing the necessary cues and allowing cells and tissues to control the ultimate shape of the engineered tissue and associated vasculature is direct fabrication of functioning tissue. Different strategies for improving vascularization of TECs. (a) Scaffold design. Panel (i) shows a scaffold with meso-scale porosity (250 μm) in which cells can proliferate and deposit matrix, while in panel (ii) the same scaffold (enlarged depiction) has added macro-scale channels (visualized in red) of 600 μm. Partly adapted, with permission, from Elsevier (2010) [152]. (b) In vivo prevascularization. An artery and a vein were joined via a loop, which was then placed in a chamber and implanted, resulting in a highly vascularized construct that was obtained eight weeks after implantation. Tortuous foam scaffolds not fabricated by AM show tissue necrosis in small and poorly connected pores at a distance from the loop. Reproduced with permission, from Elsevier (2006) [153]. (c) Growth factor delivery. Fibrin gel matrices were placed on a chicken chorioallantoic membrane. Panel (i) shows the effects of freely diffusible VEGF121, whereas in panel (ii) VEGF121 was released enzymatically by MMPs in a cell-demanded release, leading to a more regular organization of the vascular structures can be observed. Adapted, with permission, from Wolters Kluwer (2004) [154]. (d) In vitro prevascularization. Mouse myoblast cells (C2C12) were combined with human umbilical vein endothelial cells (HUVECs) and mouse embryonic fibroblasts (MEFs) and seeded on a scaffold (panel iii-1), resulting in the formation of a 3D prevascular network (panels i and iii-2). After implantation, the network anastomosed to the mouse vasculature (panels ii and iii-4). Partly adapted, with

permission, from Nature Publishing Group (2005) [155]. Panel d-iii and captions for (c) and (d) adapted, with permission, from Elsevier (2008) [151].

Table 1: Description of four common, commercially available AM techniques that are often employed in the preparation of scaffolds.

((insert FDM.TIF here))	**Melt extrusion/fused deposition modeling (FDM)**: By this process, thin thermoplastic filaments or granules are melted by heating and guided by a robotic device controlled by a computer, to form the 3D object. The material leaves the extruder in a liquid form and hardens immediately. The previously formed layer, which is the substrate for the next layer, must be maintained at a temperature just below the solidification point of the thermoplastic material to assure good interlayer adhesion.
((insert SLA.TIF here))	**Stereolithography (SLA)**: With this process 3D solid objects are produced in a multi-layer procedure through the selective photo-initiated cure reaction of a polymer. These processes usually employ two distinct methods of irradiation. The 1st method is a mask-based method in which an image is transferred to a liquid polymer by irradiating through a patterned mask. The 2nd method is a direct writing process using a focused UV beam produces polymer structures.
((insert 3DP.TIF here))	**3-Dimensional printing (3DP)**: The process deposits a stream of microdroplets of a binder material over the surface of a powder bed, joining particles together where the object is to be formed. A piston lowers the powder bed so that a new layer of powder can be spread over the surface of the previous layer and then selectively

	joined to it. The process is repeated until the 3D object is completely formed.
((insert SLS.TIF here))	**Selective laser sintering (SLS):** This technique uses a laser emitting infrared radiation, to selectively heat powder material just beyond its melting point. The laser traces the shape of each cross-section of the model to be built, sintering powder in a thin layer. After each layer is solidified, the piston over the model retracts to a new position and a new layer of powder is supplied using a mechanical roller.

Table 2: Characteristics of AM techniques that are used for the preparation of cell-laden constructs and cell-free scaffolds for tissue engineering. Processing modes are indicated by 't' for thermal processing, 'c' for chemical, 'm' for mechanical and 'o' for optical, where modes in brackets are optional.

technique	processing modes	accuracy (μm)	materials	cells	advantages	disadvantages	Refs
inkjet printing (thermal or piezo-electric)	t/m, (c)	20-100	liquids hydrogels	yes	use of existing cheap technology, multiple compositions	low viscosity prevents build-up in 3D, low strength	[28, 57-64]
3D printing	m, (c)	50	polymers, ceramics	no	multiple compositions	requires powder, cell-unfriendly environment	[65-67]
stereolithography (incl. two-photon polymerization)	o, c	0.5-50	hydrogels polymers ceramic-composites	yes	high accuracy	single composition, requires photo-curable material	[32-35, 68-70]
laser direct writing	o	20	cells in media	yes	single cell manipulation	no structural support, scalability	[49]
direct writing	m, c	1	polyelectro-lytes	not yet	high accuracy	requires solvents, cell-unfriendly environment, scalability	[71, 72]
melt extrusion (including FDM)	t, m	100	thermoplastics composites	no	technologically simple	requires strong filament and high temp.	[73, 74]
robotic dispensing	m, (t), (c)	100	hydrogels polymers ceramic-composites	yes	multiple compositions	relatively low accuracy	[36-44]
selective laser sintering	o, t	50	polymers, ceramics	no		requires powder, cell-unfriendly environment	[75, 76]
biolaserprinting	o, t	10	liquids	yes	high accuracy	low viscosity	[30,

					at high speed	prevents build-up in 3D	31]
robotic assembly	m	5	rigid solids	not yet	no heat, light or reaction required	expensive machinery	[77]

Table 3: Hydrogels used for additive manufacturing of cell-laden tissue engineering constructs.

Hydrogel	Technique	Viability	Proliferation	Refs
Natural				
Collagen	dispensing	86%	30 % in 24 h	[38]
Gelatin	disp. + aldehyde X-linking	98%	none (3 months)	[41]
Matrigel	dispensing	99%	none (2 weeks)	[42]
Agarose	dispensing	93%	none (2 weeks)	[42]
Alginate	dispensing	94%	N/A	[37]
	dispensing	91%	none (2 weeks)	[42]
Synthetic				
PEG-DA	stereolithography	65%	N/A	[34]
PEG-PPO-PEG	dispensing	84%	> 95 % cell death in 3 days	[42]
PPO-PEG-Ala-Mam[1]	disp. + UV X-linking	75%	none; after 3 days 60 % viable, up to 3 wks	[104]
PEG-HPMALA[2]	disp. + UV X-linking	94% (1d)	N/A	[36]
		85% (3d)		
Hybrid				
HA-SH + PEG-4A	gel rod deposition	≈100%	10-50 % in 4 days	[105]
Gelatin-MA + HA-MA	disp. + UV X-linking	≈100%	doubling in 7 days	[91]
Hyaluronan + dextran-HEMA	dispensing + UV X-linking	94% (1d) 75% (3d)	N/A	[44]

1 PEG-$(PPO)_2$ blockcopolymer functionalised with alanine-methacrylamide end-groups

2 PEG-(N-(2-hydroxypropyl)methacrylamide lactate)$_2$ blockcopolymer