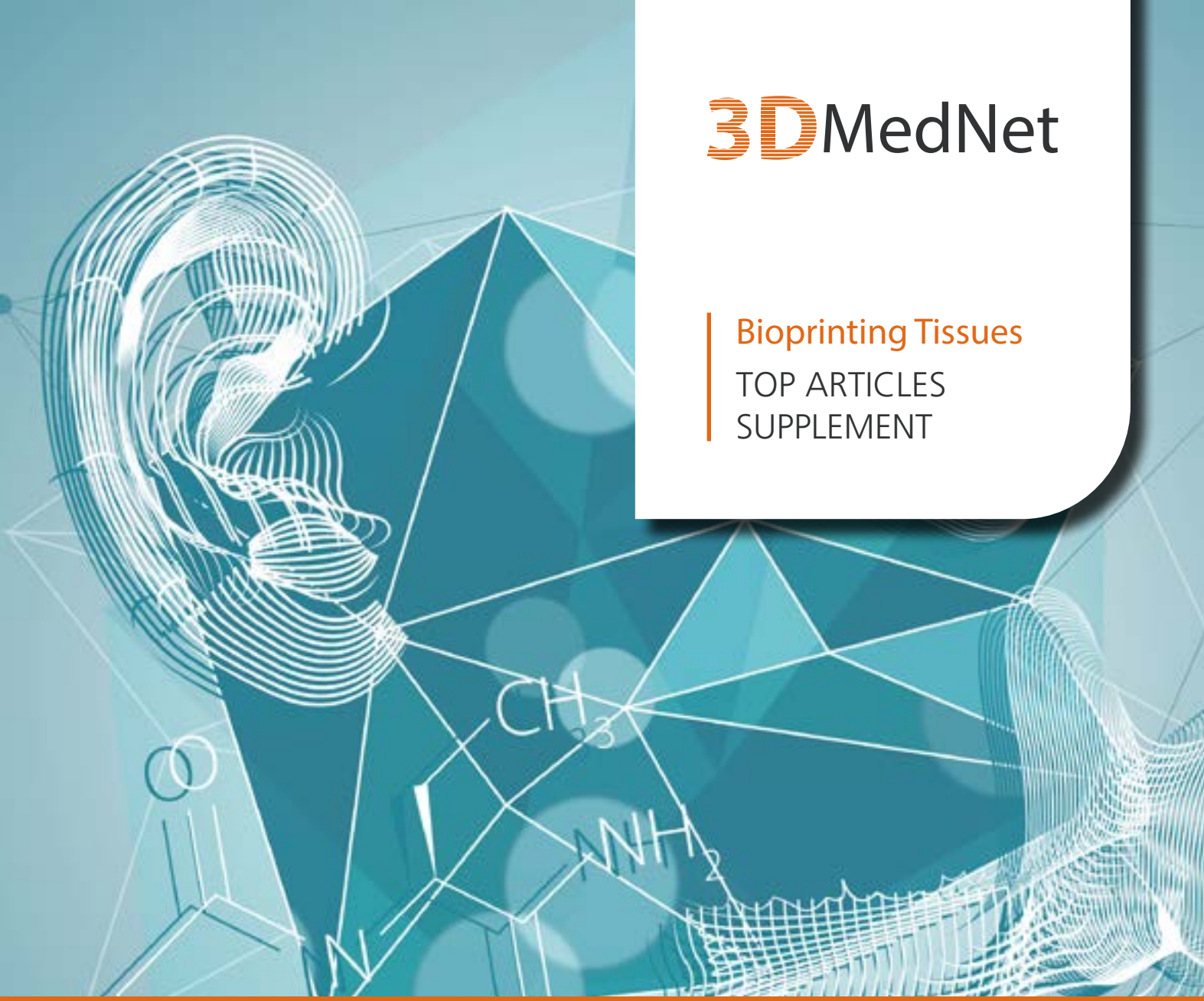

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Organ printing: promises and challenges

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Organ printing or biomedical application of rapid prototyping, also defined as additive layer-by-layer biomanufacturing, is an emerging transforming technology that has potential for surpassing traditional solid scaffold-based tissue engineering. Organ printing has certain advantages: it is an automated approach that offers a pathway for scalable reproducible mass production of tissue engineered products; it allows a precised simultaneous 3D positioning of several cell types; it enables creation tissue with a high level of cell density; it can solve the problem of vascularization in thick tissue constructs; finally, organ printing can be done *in situ*.The ultimate goal of organ-printing technology is to fabricate 3D vascularized functional living human organs suitable for clinical implantation. The main practical outcomes of organ-printing technology are industrial scalable robotic biofabrication of complex human tissues and organs, automated tissue-based *in vitro* assays for clinical diagnostics, drug discovery and drug toxicity, and complex *in vitro* models of human diseases. This article describes conceptual framework and recent developments in organ-printing technology, outlines main technological barriers and challenges, and presents potential future practical applications.

Organ printing in essence is a biomedical application of rapid prototyping technology or additive layer-by-layer manufacturing. In a more narrow sense, it could be defined as computer-aided, layer-by-layer deposition of biologically relevant materials [1,2]. The ultimate goal of organ-printing technology is to fabricate 3D vascularized functional living human organs suitable for clinical implantation. Rapid prototyping is already well established and includes many technology variants such as stereolithography, selective laser sintering (SLS), fused deposition modeling (FDP), 3D printing (3DP), ballistic particles manufacturing (BPM) and others [3]. The main challenges of biomedical applications of rapid prototyping technology are the adaptation of existing systems for specific biological materials when it is technically possible, as well as development of novel deposition systems specifically designed for biologically relevant materials. One simple way to describe the principles of emerging organ-printing technology or bioprinting is to use the analogy of traditional printing invented by Johannes Gutenberg. In order to print a book using Gutenberg's technology, it is necessary have at least five essential components: written text, a printing press, movable type, paper and ink. Similarly, in order to print a living human organ, it is necessary have computer-aided design of the desired organ (its 'blueprint' or analog of text), a 'bioprinter' or robotic dispensor (analog of printing press), a cartridge or container for dispensing biomaterials and living cells or cell aggregates (analog of movable type), processible biomimetic hydrogel ('biopaper' – analog of paper), and self-assembling cell aggregates or single cells in hydrogels ('bioink' – analog of ink). The separate development of these five most essential components of emerging organ-printing technology is already underway. However, the real challenge is to find the optimal ways of putting these technological components together into well integrated, scalable industrial technology and eventually bioprint functional living human organs suitable for clinical implantation.

It is very important to be maximally inclusive and respect the valuable contributions of all newcomers and players in any new, rapidly evolving field such as organ printing. On the other hand, certain demarcation is essential for properly defining the essential novelty of a new emerging field. This review is not focusing on recent developments in using rapid prototyping technology for fabrication of solid scaffolds [4–8], because we strongly believe that it represents important but incremental improvements of already existing and conceptually traditional solid scaffold-based tissue engineering, that is, it does not have the revolutionary potential offered by 'true' or narrowly

Keywords: bioprinter, organ printing, tissue engineering, tissue fusion, tissue spheroids

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defined organ bioprinting technology. Thus, we will focus our attention on the limited number of papers that we believe represent important milestones and reflect essential and novel characteristics specific for the emerging organ printing concept.

Recent developments in organ printing

Traditional or classic tissue engineering is based on fabrication of porous solid biodegradable scaffolds with sequential cell seeding in bioreactors. The main rationale behind this approach is a need to maintain, at least initially, the shape and mechanical properties of the tissue engineered construct and to provide a substrate for cell attachment and signals for cell differentiation and tissue development [9]. The main limitations of the solid scaffold approach are the low level of precision in cell placement, especially when engineering multicellular constructs [10], an intrinsic problem with vascularization of thick tissue constructs, and the extremely laborious, slow and costly nonautomated tissue assembly process [11]. Organ printing or robotic biofabrication offers an interesting alternative to solid scaffold-based tissue engineering. Michael Sefton and his coworkers in a recent *Proceedings of the National Academy of Sciences* paper and series of other excellent publications wrote [12–14]:

'Modular tissue assembly is a biomimetic alternative to traditional scaffold-based strategies, which offers many advantages for engineering whole-organ and large-tissue grafts and potentially transforms the conventional cell seeding/porous scaffold paradigm of tissue engineering'.

The journal *Tissue Engineering* published an excellent review by Federovich *et al.*, who also claimed that [15]:

'Layered deposition of cells and cell aggregates using various rapid prototyping (RP) techniques confers reproducible control over cell placement, surpassing uneven, random, and slow cell distribution within the scaffold, and yields a defined scaffold structure with regard to external shape and internal morphology'.

What are the principle differences between solid scaffold-based tissue engineering and robotic biofabrication and why do some people state that the tissue self-assembly approach or organ printing is an emerging transforming technology, which has potential for surpassing traditional solid scaffold-based tissue engineering? We can formulate several main advantages of the emerging technology of organ printing. First, organ printing is an automated approach that offers a pathway for scalable reproducible mass production of tissue engineered products from standardized modular building blocks – an essential feature for successful commercialization [1]. Second, organ-printing technology allows a high level of control in the placement and 3D precision positioning of several cell types due to a precise control position of the dispensor nozzle in the X-Y-Z coordinate [1]. Third, organ printing allows one to create tissue with a high level of cell density, especially when using cell aggregates as the modular building blocks or bioink [10]. Fourth, organ-printing technology has the potential to solve the problem of vascularization in thick tissue constructs through simultaneous deposition and printing of the vascular tree within the bioprinted tissue construct [11]. Finally, some tissue engineers strongly believe that organ printing can be done *in situ* [16], and that the bioprinter or dispensing devices can eventually evolve into some sort of novel surgical tools for *in vivo* tissue building, which could revolutionize surgical practice [16]. The emergence of advanced organ-printing technology does not mean that classic solid-scaffold approaches have no future; on the contrary, they will continue to be developed and eventually be integrated into newer, more advanced tissue engineering technologies. Moreover, rapid prototyping can create even more sophisticated solid scaffolds for traditional tissue engineering. However, this review is focused mainly on organ-printing technology defined in narrow terms. Broad potential biomedical applications of existing rapid prototyping technology for printing and fabrication nonbiodegradable external prostheses, devices and implants, as well as biodegradable solid scaffolds for orthopedics and craniofacial applications, can be found elsewhere [4–7,17–19].

One of the most well developed rapid prototyping technologies that can be adapted for organ printing is stereolithography. There are several partly successful attempts to use photosensitive hydrogels with living cells for rapid fabrication of 3D tissue constructs with desirable geometry [20–22]. However, cell density and viability were far from an optimal and desirable level. A geometrical tube, fabricated with a very low density of viable functional cells, without

any sign of organized histotypical extracellular matrices, is an important step in the right direction, but it is obviously not even close to an authentic blood vessel. Most importantly, capacity for precise patterning of different cell types in the horizontal direction was rather limited. The technology was dramatically improved after employing a combined dielectrophoresis with microfabrication approach by the Massachusetts Institute of Technology (USA) group of Sangeeta Bhatia, allowing improved cell patterning and increased cell density by horizontal control of cell placing [23]. Synthesis of more cell-friendly, biomimetic, photosensitive hydrogels containing arginine-glycine-aspartic acid (RGD) peptide specific for cellular integrins was another important advancement for improving cell viability [21]. As result of technological improvements, biofabrication of a 3D liver construct with superior functionality compared with a 2D liver cell monolayer have been reported [21,23]. The absence of vascularization and potential problems with scalability of this approach remain arguably problematic, but this Federation of American Societies for Experimental Biology paper definitely represents one of the most dramatic recent developments in robotic tissue microfabrication.

The bioprinting group from Tsinghua University were able to print viable 3D liver tissue constructs expressing certain liver-specific functionality and suitable for superfusion (not intravascular perfusion yet) using an original robotic dispensing system and mixture of liver hepatocytes with chitosan–collagen hydrogel. What is most interesting is that after incubation the cell density, initially relatively low, was increased as a result of hydrogel degradation, cell proliferation or a combination of both these processes (authors did not specify potential mechanism) [24,25]. The precise placing of different cell types and tissue-specific patterning, as well as vascularization, remained unsolved issues. This group is actively working on solving 3D tissue construct vascularization by building a cellularized branched vascular tree scaffold and replacing avascular superfusion with intravascular perfusion. The seamless connection of such a branched macrovascular cellularized solid scaffold with a bioprinted microvascularized 3D liver tissue construct will be a challenge. Robotic dispensing of cells and hydrogel mixture was recently also used for printing a 3D cartilage tissue construct, achieving desirable geometrical shape and cell viability but still unproven tissue functionality and biomechanical properties [26]. One obvious advantage of focusing on bioprinting cartilage tissue constructs is that cartilage is avascular tissue.

Michael Sefton's group from the University of Toronto, Canada, employed an original and innovative approach to solve the problem of vascularization of tissue constructs created from a modular tissue block. Encapsulated in collagen, liver cells in the form of rod-like tissue constructs were fabricated by extrusion and cutting and then coated with endothelium. These endothelialized liver rod-like tissue constructs were placed by dense packing into a perfusion tube and thus provide endothelialized channels for blood perfusion. Authors logically extrapolated that 'the next step is exploiting the modular concept in a form that is suitable for *in vivo* use (e.g., adding components to enable anastomoses to the host vasculature; using biocompatible components) and understanding how the modular construct and the endothelial cell-lined channels remodel once implanted' [13,14,27].

Using cell aggregates and tissue spheroids as modular building blocks for organ-printing technology was originally presented in a series of publications from our labs and those of our colleagues [1,10,28–31]. It was shown that cell aggregates behave as viscoelastic fluid and have the capacity to fuse in permissive hydrogels through the tissue fusion process [10]. Tissue fusion is a ubiquitous process during embryonic development [32] and constitutes the biological foundation for organ-printing technology. An independent study published recently confirmed our original observations [33]. The series of elegant studies, conducted by a tissue engineering group in Switzerland, confirmed that cell aggregates can fuse into larger tissue constructs [34,35]. The original criticism surrounding the use of tissue spheroids as building blocks in organ-printing technology, based on the assumption that tissue spheroids are too large for effective vascularization, was overcome by demonstrating that single-tissue spheroids can be microvascularized [36]. Finally, it has been demonstrated that endothelialized vascular tissue spheroids, as well as unilumenal vascular tissue spheroids, can be used as building blocks for bioprinting intraorgan branched macrovascular trees [1,10,37]. Lumenized tissue spheroids and cyst-like spheroids can also be used as building blocks for designing kidney epithelial tubes [38].

Thus, at least three realistic competing approaches for using robotic cell placing in organ bioprinting technology have emerged:

- Precise placement of single cells using inkjet printing [39,40] or stereolithography [20–22];
- Cell–hydrogel mixtures for bioprinting cellular tissue constructs [24,25];
- Dispensing of high-density tissue spheroids or cell aggregates as building blocks [10,28,29,31].

All of these approaches have equal potential and deserve to be systematically explored. However, it remains to be seen which of these approaches will be the most effective approach in the evolving technology of organ bioprinting. We are skeptical regarding the potential of using rapid prototyping for solid biodegradable scaffold fabrication because conceptually it still belongs to the domain of traditional solid scaffold tissue engineering, which has intrinsic limitations. We, as well as some pioneers of these technologies, do not consider certain innovative, but not scalable, laser-based tissue assembly technology approaches (such as laser-guided direct writing) [41,42] as significant developments or milestones towards development of organ printing. It does not mean that these technologies could not find interesting biomedical applications. It simply means that they are not scalable. We also do not believe that hydrogel-free bioprinting using a cell suspension in an inkjet printer (single cell in single drop) will allow one to create a 3D human organ. However, we would like to be wrong in our subjective evaluations. Only the future can show which technology will really work and which technology will represent nothing more than just simple noise, distraction or technological dead end. A diversity of approaches and strong competition among different approaches is probably the best guarantee for the emergence of the most effective variant of organ-printing technology. Regardless of which organ-printing technology emerges as the most effective or industry standard, it must solve several challenges, which we will try to outline in the next section. Most importantly, it must be computer-aided, scalable, automated technology that will allow rapid robotic assembly of 3D vascularized and intravascular perfused functional human organs.

Box 1. Characteristics of ideal hydrogel for organ printing.

- Bioprocessible (dispensible and fast solidification)
- Biomimetic (functional arginine-glycine-aspartic acid peptides for improving viability)
- Biocompatible (nontoxic, high cell viability)
- Intelligent (stimuli-sensitive)
- Tissue fusion permissive (optimal physicochemical properties)
- Shape maintanence (preventing construct melting and distortion)
- Hydrophylic (efficient diffusion)
- Biodegradable (removable on demand)
- Naturally derived hydrogels (collagen, fibrin, hyaluronan based)
- Pro-angiogenic and loaded with survival and angiogenic factors (enhancing bioprinted construct viability)
- Affordable (relatively low cost)
- FDA approvable (noncancerogenic and nonimmunogenic)

Top ten challenges in organ printing technology

The general challenges in the field of tissue engineering are well known and clearly outlined in several excellent, insightful publications [43,44]. Here, we will focus our attention on ten specific challenges for emerging organ technology.

Organ blueprint

The 'organ blueprint', especially in 'bioprinter friendly' stereo lithography (STL) format, is basically a software-based computer program providing detailed instruction for layer-by-layer placement of specific biocomponents using a dispensing device in accordance with the original computer-aided design (CAD). The main challenge for organ blueprint design is postprocessing fusion, retraction, remodeling and compaction of the printed soft-tissue construct [10,33]. Thus, in order to get the desirable mature organ size and shape, the organ blueprint must be larger and probably have a slightly different shape. CAD must include experimentally estimated and validated coefficients of specific tissue compaction, retraction and remodeling. CAD or blueprints for 3D soft-organ printing could not be automatically derived from a 3D clinical imaging file, as is the case for CAD for solid organ scaffolds, because bioprinted tissue constructs and bioprinted organs are soft tissues and they are subject to postprinting remodeling associated with tissue fusion, tissue compaction and tissue maturation processes [10,29].

Figure 1. Robotic bioprinters.

(A) Bio-Assembly Tool (Sciperio/nScript, USA). **(B)** Bioplotter (Envisiontech, Germany). **(C,D)** Robotic dispensors (Neatco, Canada).

In silico *tissue self-assembly*

Decoupling of design and fabrication is one of the main principles of engineering [15,16]. Detailed computational simulation of the tissue self-assembly process based on predictive mathematical modeling and packing theory is a prerequisite for organ printing. Initial data strongly demonstrate that this is not only a desirable goal but also a doable task [37]. Moreover, *in silico* tissue assembly is necessary for designing mechanical engineering aspects of the entire robotic biomanufacturing process. So-called computational tissue engineering is still focusing predominantly on CAD of rigid solid scaffolds [4,5,7]. Thus, computer simulation of dynamic tissue self-assembly and postprocessing remodeling of bioprinted 3D soft tissue constructs are important tasks for the rapidly evolving field of computational tissue engineering.

Design of biofabrication process

It is becoming increasingly obvious that fabrication of complex 3D organs such as the kidney will require several steps and a broad spectrum of specially designed equipment. The future of an organ printing plant will likely resemble car assembly or airplane assembly plants. Modern software will allow one to design the whole organ biomanufacturing process and the corresponding robotic biofabrication equipment, as well as sequential and/or parallel fabrication steps. It is one of the most challenging tasks for mechanical engineers involved in the development of organ-printing technology.

Biopaper

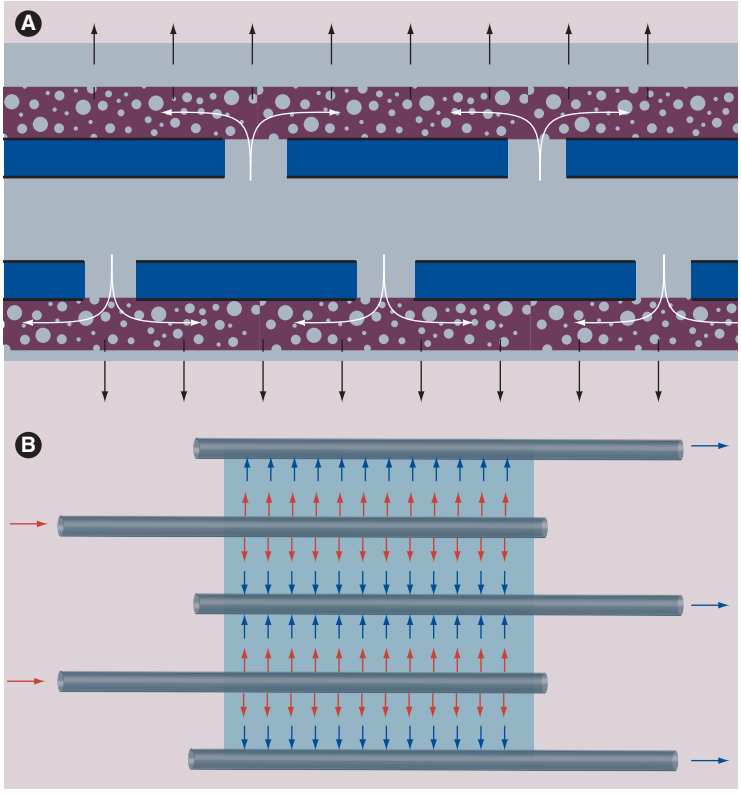
Biopaper can be defined as processible and biomimetic tissue fusion-permissive hydrogels specially designed for the bioprinting process. The

first comprehensive review regarding hydrogels as extracellular matrices for organ printing was recently published [15]. Criteria for ideal hydrogels for organ-printing technology are summarized in Box 1. Chemical engineers have unique opportunities to use their professional expertise for the design and synthesis of a battery of bioprocessible and biomimetic hydrogels or extracellular matrices suitable for organ-printing technology. For example, synthesis and emplyoment of biomicking photosenstive hydrogel incorporating functional RGD peptide dramatically improved viability of printed tissue construct [21]. Design and synthesis of processible and biomimetic hydrogels (biopaper) represents one of the most important and challenging tasks in development of organ-printing technology [15].

Bioink

We define 'bioink' as standardized modular tissue and organ building blocks. The fundamental biological principle of organ-printing technology is the tissue fusion process. The tissue fusion process employed in organ-printing technology is a recapitulation or utilization of ubiquitous tissue-fusion process occurring during embryonic development [11,32]. The large-scale fabrication of self-assembled tissue spheroids with viscoelastic, fusogenic, fluid-like properties is essential for reproducible organ printing [1,2,29]. Although small-scale fabrication of tissue spheroids and cell aggregates is a well established process with different approaches such as hanging drop, shaking, centrifugation and cutting, extrusion and cutting, using nonadhesive substrates [14,45–47], and many other techniques, scalable fabrication of standardized tissue spheroids suitable for robotic dispensing is still an important challenge in development of organ-printing technology. Designing cartridges for bioink is another serious challenge.

Figure 2. Design of dripping irrigation system for triple perfusion bioreactor.

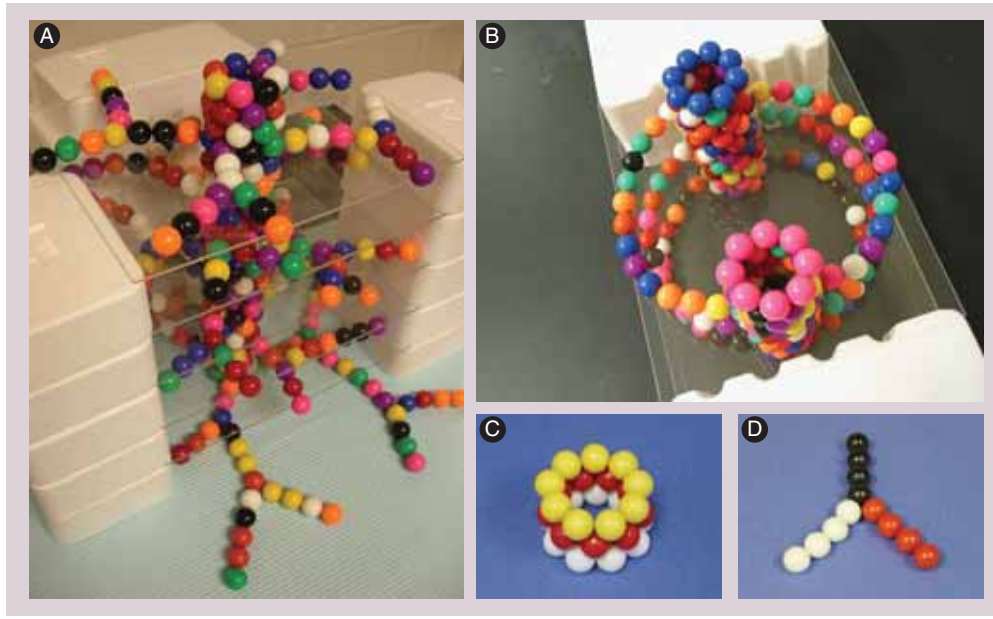


(A) Scheme of dripping irrigation system. Metallic needle with machined microholes coated with porous polyurethane. **(B)** Scheme of flow direction in tissue blocks of printed tissue construct.

Bioprinters

Design and fabrication of the bioprinter or robotic dispenser and a biologically friendly rapid prototyping machine are important challenges for mechanical engineers involved in the development of organ-printing technology and adaptation of existing rapid prototyping technologies for bioprinting and biofabrication. Some already existing commercial and experimental devices for bioprinting are presented in Figure 1. Sciperio/nScript Inc (Orlando, FL, USA) is probably the only commercial entity that has seriously focused on designing industrial bioprinters (BioAssembly Tool) (Figure 1A) for organ printing. Envisiontech's Bioplotter (Figure 1B) is one of the first commercially available bioprinters that enables bioprinting of 3D living tissue using a mixture of cells with fibrin hydrogel [48]. Together with Canadian company Neatco we designed several simple robotic bioprinters (Figure 1C & D). Organ bioprinting can also be considered as an integral part of the ongoing desktop manufacturing revolution. Some engineers define a desktop rapid prototyping system as a 'personal fabricator', analogous to a personal computer. A group at Cornell University designed the first affordable, easy to assemble personal fabricator [26]. If mass produced, it was predicted that the price of a personal fabricator could be as low as US$250. It has already been shown that this personal fabricator can be used for rapid prototyping of tissue-engineered cartilage [26].

Figure 3. Design of branched vascular tree using vascular tissue spheroids.



(A) Assembly of branched vascular tree. **(B)** Assembly of circulatory arterial-venous vascular perfusion unit. **(C)** Assembly of vascular tube from vascular tissue spheroids. **(D)** Assembly of elementary 'Y-shape' branched vascular unit.

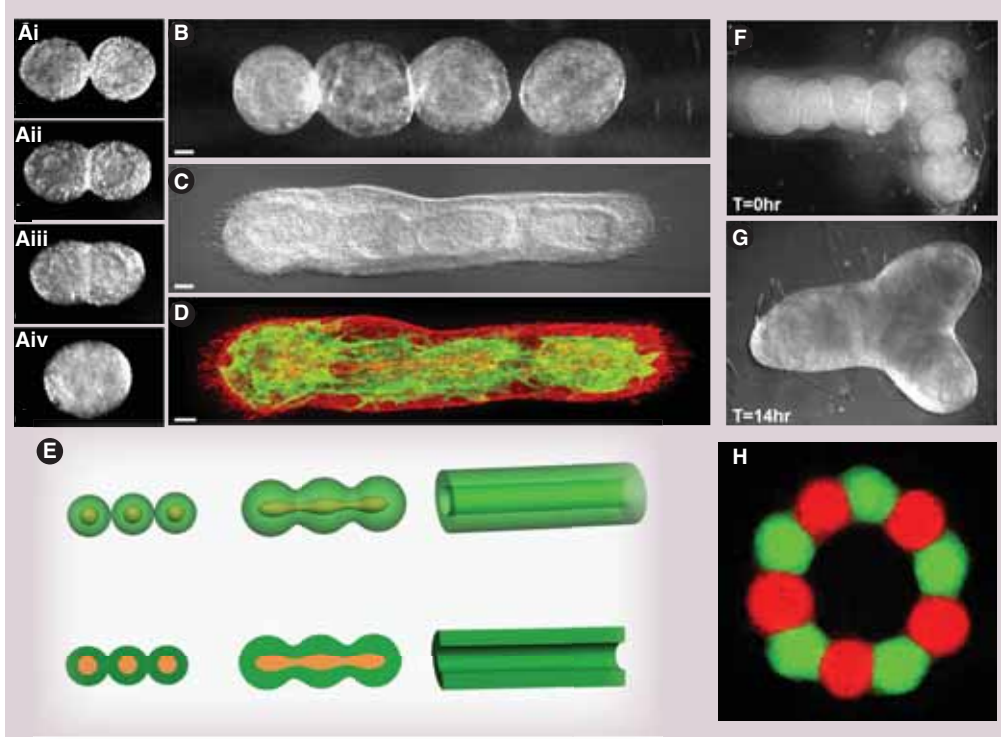
Bioreactors

Bioreactors are one of the enabling tools in the field of tissue engineering. However, bioreactors for bioprinted 3D thick tissue constructs must have certain essential characteristics different from bioreactors used in traditional tissue engineering. First, it must be a perfused bioreactor that will allow perfusion of the intraorgan branched vascular tree. Second, it must provide a temporal, removable irrigation system that will 'buy' necessary time until the bioprinted intraorgan branched vascular system becomes mature and functional enough for initiation of intravascular perfusion. The development of a novel type of irrigation perfusion bioreactors based on using temporal, removable, porous needles with pressure-controlled, dripper-like systems is essential for maintaining viability of printed organ (Figure 2). The experimental system for testing perfromance of needle-based bioreactors has been recently developed [49]. Third, it must provide dynamic biomechanical conditioning for accelerated tissue maturation during postprocessing [50]. Finally, the bioreactor must be seamlessly integrated with the bioprinter or rapid prototyping machine and allow easy placing and damage-free removal of bioprinted tissue constructs in sterile wet conditions.

Viability & vascularization

The viability of printed tissue constructs include several aspects: preprocessing cell survival during loading of bioprinter cartridges, cell survival during processing [51], and tissue construct survival during postprocessing. The last challenge can be addressed by a combination of several technological approaches: rapid assembly of a perfusable branched vascular tree, using special hydrophilic hydrogels loaded with survival factors coupled with a special bioreactor with temporal removable irrigation system and, finally, by precisely controlling the tissue compaction process and construct diffusion properties. Simultaneously printing the organ with a 'built-in' intraorgan branched macrovascular tree is probably the most challenging engineering task. However, our preliminary data strongly suggest that it is technically feasible (Figure 3 & 4). There are also several evolving approaches for microvascular bed self-assembly when using endothelialized and microvascularized tissue spheroids as building blocks in organ-printing technology (Figure 5). The relative effectiveness of these approaches in ensuring adequate perfusion and viability of bioprinted 3D thick tissue constructs and organs remains to be demonstrated.

Figure 4. Bioassembly of vascular unit.



(A) Sequential stages **(i–iv)** of fusion of two vascular tissue spheroids in hanging drop. **(B–D)** Sequential stages of tissue fusion of four vascular tissue spheroids placed in collagen hydrogel. **(E)** Scheme of linear vascular tube bioassembly from unilumenal vascular tissue spheroids. **(F,G)** Sequential stages of bioassembly of unilumenal vascular tissue spheroids into branched unit. **(H)** Bioassembly of vascular ring from human vascular tissue spheroids.

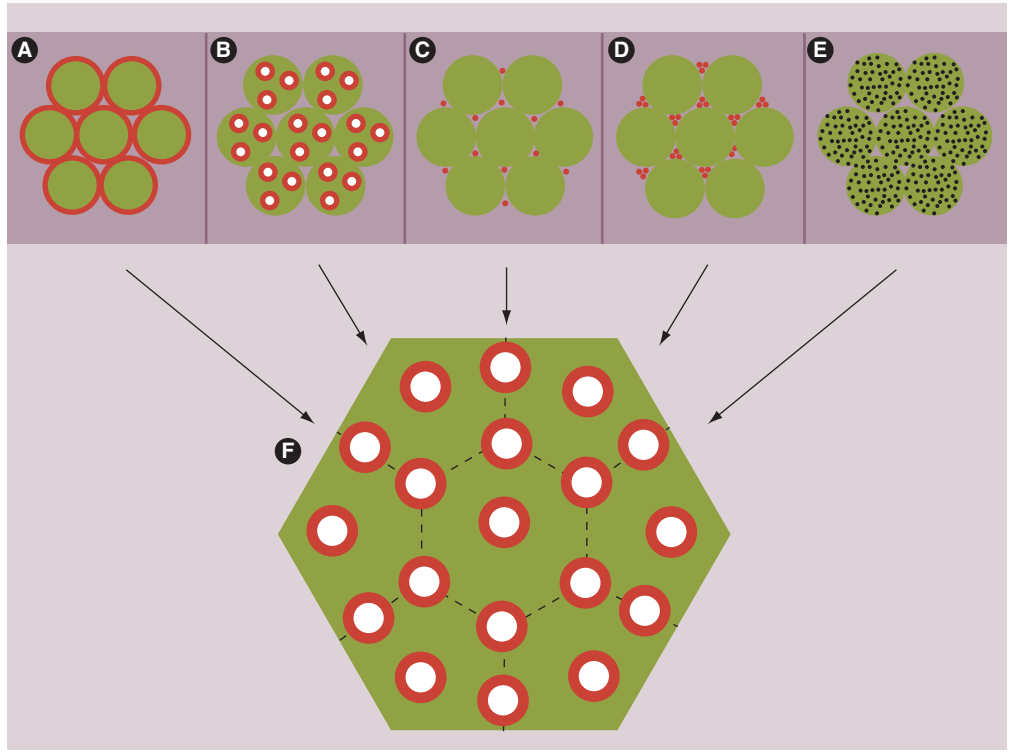
Accelerated tissue maturation

Owing to the fluidic nature of additive biomanufacturing processes and the absence of solid scaffolding, accelerated tissue maturation is one of the most important biological challenges of organ-printing technology. Bioprinting technology is based on the assumption that precisely placed cell populations at high density can rapidly form and assemble authentic tissues through cell adhesion, cell sorting and tissue fusion processes, and then start to synthesize the tissue- and organ-specific extracellular matrices, which will provide and maintain the desirable geometrical shape and mechanical properties of the organ. Identification of biologically effective and economically reliable accelerated tissue maturation procedures and so-called 'maturogens', or physical, chemical and biological factors that accelerate postprinting or postprocessing tissue maturation and assembly [52], is not only essential and integral, but also probably the most challenging part of organ-printing technology development.

Noninvasive biomonitoring

Development of noninvasive, nondestructive quantitative methods and biosensors for monitoring the kinetics of postprocessing tissue self-assembly, remodeling and maturation is another important challenge. It includes development of objective and reliable criteria or 'tissue maturation biomarkers' for achieving sufficient levels of tissue maturation and organ functionality using genomic and proteomic technologies. Optical, biomechanical and physical methods, as well as biochemical analysis of perfusate fluid, could be used for nondestructive biomonitoring of tissue maturation and for identification of structural and functional tissue maturation biomarkers. A combination of predictive mathematical models and computer simulations as a reference point with real-time registration of tissue maturation biomarkers will provide an intelligent and automated tissue maturation biomonitoring system.

Figure 5. Design of printed tissue constructs microvascularization.



(A) Endothelized tissue spheroids (spheroid coated with endothelium). **(B)** Vascularized tissue spheroids (spheroid with microvessels inside). **(C)** Tissue spheroids with endothelial spheroids between. **(D)** Tissue spheroids and isolated endothelial cells in hydrogel between. **(E)** Cell aggregates with unsorted endothelial cells and organospecific cells. **(F)** Microvascularization of bioprinted tissue construct after fusion of tissue spheroids (green: organospecific tissue spheroids; red: endothelial cells, dark pink: hydrogel).

Practical applications of bioprinting

There are several potential biomedical applications of bioprinting technology. Biopatterning of 2D cell-based *in vitro* assays can create cell-based assays for cellomics and high-throughput and high-content drug discovery and drug toxicity assays. More complex printed 3D patient-specific tumor assays could be, at least theoretically, more predictive and could improve effectiveness of antitumor therapy. Bioprinted, complex, authentic, 3D human tissue-based *in vitro* drug discovery and drug toxicity assays can be potentially more predictable than small or even large animal testing. It can dramatically reduce the costs of drug development and improve drug safety. 3D human tissue-based *in vitro* assays can also be used as models of human disease both for basic and applied therapeutic research. *In vitro* robotic biofabrication of organ printing from autologous cells can make allogenic organ transplantation obsolete and once and forever eliminate patient waiting lists for organ transplantation. Recent medical economic studies demonstrated that if kidneys sales were allowed, potential vendors could charge $250,000 for one kidney and still have monetary savings for healthcare providers [53]. The number of patients with chronic kidney disease waiting for kidney donors in 2010 will reach 100,000. Thus, the potential market for bioprinted human kidneys alone is $25 billion. *In situ* robotic biofabrication of tissue and organs can revolutionize and reinvent surgery [16]. Some tissue engineers are seriously considering this direction [16].

Future perpective

Organ printing is a novel transforming approach in tissue engineering, which has a potential for surpassing traditional solid scaffold-based tissue engineering. The history of technology development teaches us that standardization, automation and robotization is only one economically feasible pathway towards mass industrial production and effective commercialization. The

focus of leading-edge tissue engineering research is already moving into the area of directed tissue self-assembly and it will further move into the sphere of robotic biofabrication and organ-printing. The main practical outcome of investment into development of organ-printing technology will be an industrial-scale robotic biofabrication of complex human tissues and organs.

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No writing assistance was utilized in the production of this manuscript.

Executive summary

What is organ printing?

- Organ printing is a biomedical application of rapid prototyping technology.
- Organ printing is a computer-aided, layer-by-layer, robotic additive biofabrication.
- Organ printing is a transforming technology, which has potential for surpassing traditional solid scaffold based approach in tissue engineering.
- Organ printing is info-robo-nano-bio technology.

Preprocessing, processing & postprocessing

- Preprocessing (development of computer-aided design or organ blueprint).
- Processing (actual printing using bioprocessible hydrogel and bioink or self-assembling tissue spheroids).
- Postprocessing (tissue fusion, remodeling and accelerated tissue maturation).

Blueprint, bioprinters, bioink, biopaper

- Blueprint is a human organ computer-aided design in stereo lithography file.
- Bioprinter is a computer-based robotic dispensor device.
- Tissue spheroids (bioink) are modular building blocks in organ-printing technology.
- Biopaper is a bioprocessible and biomimetic hydrogel suitable for bioprinting.

Tissue fusion & accelerated tissue maturation

- The main biological principle of organ-printing technology is a tissue spheroid fusion or directed tissue self-assembly.
- The accelerated tissue maturation is postprocessing conditioning of printed tissue construct towards a desirable level of functional maturity.

Main challenges in organ printing

- Designing organ blueprint.
- Development of multifunctional bioprinters.
- Synthesis of bioprocessible and biomimetic hydrogel.
- Postprinting accelerated tissue maturation.

Conclusion

- In the short term, organ-printing can be used for biofabrication *in vitro* model for drug toxicity, drug discovery and modeling human diseases.
- In the long term, organ-printing technology can solve the problem of human organ shortage for transplantation once and forever.

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Review

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Regenerative Medicine

Recreating composition, structure, functionalities of tissues at nanoscale for regenerative medicine

Nanotechnology offers significant potential in regenerative medicine, specifically with the ability to mimic tissue architecture at the nanoscale. In this perspective, we highlight key achievements in the nanotechnology field for successfully mimicking the composition and structure of different tissues, and the development of bio-inspired nanotechnologies and functional nanomaterials to improve tissue regeneration. Numerous nanomaterials fabricated by electrospinning, nanolithography and self-assembly have been successfully applied to regenerate bone, cartilage, muscle, blood vessel, heart and bladder tissue. We also discuss nanotechnology-based regenerative medicine products in the clinic for tissue engineering applications, although so far most of them are focused on bone implants and fillers. We believe that recent advances in nanotechnologies will enable new applications for tissue regeneration in the near future.

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Regenerative medicine aims to restore the function of human tissues and organs by stimulating the intrinsic regenerative capacity of the body by utilizing cells, biomaterials and growth factors [1,2]. Current advances in regenerative medicine have led to the creation of bioengineered tissues and organs that can perform key biological functions. For example, biomimetic tissues including bone, blood vessels, urethra, skin, liver, lung, bladder and trachea transplants have been successfully engineered and implanted *in vivo* [3–10]. Bioengineered tissue constructs can grow and remodel *in vivo* since they are composed of living cells, or can stimulate body cells to migrate and integrate into scaffolding materials.

Currently, by virtue of recent achievements in nanotechnology, the composition and structure of bioengineered tissues are becoming more analogous to natural tissues at the nanoscale, providing a biomimetic niche for cells. The activities of cells depend on biochemical and physical signals from surrounding tissues, and since cells dynamically interact with their local microenvironment at the nanoscale, it is necessary to control properties of engineered tissues at these scale lengths. In addition, nanostructured biomaterials can decrease inflammatory response and increase wound healing in comparison to conventional biomaterials, possibly due to their high surface energy affecting protein adsorption and cell adhesion [11]. In this sense, advanced nanotechnologies for mimicking native tissues can also overcome the disadvantages of using autografts or allografts, such as the risk of immune reaction, infection and disease transmission.

In this paper, we highlight key achievements in the nanotechnology field to recreate the composition, structure and functionality of major tissues and organs, using bio-

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mimetic and bio-inspired approaches to improve tissue regeneration. In addition, we report on clinically approved nanotechnology-based regenerative medicine products for tissue engineering applications. By providing an overall view of the recent status of nanotechnology applications in the regeneration of various tissues, we expect that this article will be particularly helpful for those who are investigating the regeneration of complex tissues.

Biomimicking tissue composition at nanoscale

Every tissue in the body has its own nanoscale composition which provides a suitable microenvironment to direct cellular differentiation toward a particular lineage. Since engineered nano-architecture features a high surface area to volume ratio, it can systematically expose cells to multiple biological components with different functionalities. The ability to control the spatial distribution of materials at the nanoscale can also enhance tissue regeneration by enabling better integration with host tissue [12]. For example, bone tissue is mainly composed of inorganic calcium phosphate nanocrystals and organic components (mainly collagen type I) [13–15]. It is reported that a nanocomposite scaffold that is composed of both organic and inorganic components of bone tissues can promote bone regeneration [16,17]. In addition, the inorganic phase of human bone tissue is composed of two major bone minerals: hydroxyapatite (HAP: $Ca_{10}[PO_4]_6[OH]_2$) and whitlockite (WH: $Ca_{18}Mg_2[HPO_4]_2[PO_4]_{12}$) nanocrystallites, with different physicochemical properties [14,15]. For example, Mg^{2+} ions are too small in size to maintain a HAP crystal structure, and so are mostly incorporated in the WH crystal structure [14,18]. Furthermore, it is reported that these two bone crystals are distributed in different ratios depending on certain regions of bone tissue [14], implying that HAP and WH have distinguished biological roles. Therefore, controlling their spatial distribution at the nanoscale is important for mimicking native bone tissue.

In Table 1, we have listed representative examples of recent research achievements to recreate the nanoscale composition of each tissue type. However, despite many outstanding achievements in both the nanotechnology and tissue engineering fields, so far, most bioengineered tissues are still dependent on the usage of bulk materials with micrometer scale designs or larger, which have limited tissue functions. Therefore, there remains a strong need to further develop nanomaterials that mimic the major components of tissues at the nanoscale and apply them for tissue regeneration.

Mimicking nanoscale tissue structure

Human tissues have complex topographical features at the nanoscale that can physically influence the behavior of cells by directly modulating their migration, orientation, differentiation and proliferation. For example, skeletal and cardiac muscles are composed of perpendicularly interwoven collagen strips and elastin bundles at the nanometer scale [28]. Also, bone tissue is composed of HAP nanocrystals that form nanopatterns along collagen fibers [29]. In addition, highly connected nanopores/channels in tissues can continuously supply a sufficient level of oxygen and nutrients to cells, and allow for intercommunication between different cell types. For example, there exist three levels of hierarchical pore architectures within cortical and cancellous bone, ranging from 10 to 20 μm in radii, which support blood or interstitial fluid transportation [30].

To mimic the nanoscale structure of each tissue type to stimulate cells with the proper topographical cues, nanofibrous and nanocomposite structures, nanoscale surface topographies and nanoporous/nanochannel networks in the scaffold have been engineered by nanotechnologies such as electrospinning, nanolithography, self-assembly, phase separation and sacrificial template methods (Table 2).

Since the cellular microenvironment includes ECM components such as fibril structured proteins and polysaccharides [43], engineered nanofiber networks can support cellular growth and regulate cellular behaviors in a physiologically similar manner [44]. Aligned nanofibers are especially useful in guiding cellular orientation to mimic the anisotropy of natural tissues, including heart, nerve, tendon and blood vessels. For example, when human tendon progenitor cells were seeded on aligned poly (L-lactic acid) nanofibers that recapitulated parallel collagen fibers in tendon, these cells expressed higher level of tendon specific genes compared with cells grown on random fibers [34].

Nanocomposite structures are used widely, as they can enhance the mechanical strength of hybrid organic/inorganic composites, and thus influence cellular proliferation and differentiation. To mimic the organization of bone tissue that is composed of inorganic minerals and organic collagen matrix, silicate nanoparticles were incorporated into organic materials, enhancing mechanical properties (i.e., compressive strength, tensile strength and elastic modulus) and further promoting cellular proliferation [37,38,45]. In fact, stiffness is one of the key parameters for altering cell growth and differentiation [46,47]. Recently, Alakpa *et al.* fabricated supramolecular nanofiber hydrogels and controlled their stiffness to direct the differentiation of stem cells without any biochemical functionalization [47].

Table 1. Examples of biomimicking composition of tissues at nanoscale.

Tissue	Nanotechnologies	Functionality	Tissue regeneration capacity	Ref.
Bone	Hydroxyapatite composite sponge with concentrated collagen nanofibers	Mimicking bone chemistry based on osteoconductive scaffolds composed of inorganic material and natural polymers	Induced continuous deposition of lamellar bone tissue while maintaining osteoblast activity	[17]
	Synthesis of the two major bone crystals: hydroxyapatite and whitlockite nanoparticles	Mimicking inorganic composition of bone, providing mechanical stability and stimulating osteogenic differentiation of stem cells	Enhanced proliferation and differentiation of bone cells and induced rapid regeneration of bone tissues	[19,20]
	Self-assembled peptide amphiphile nanofibrous matrices to induce biomimetic nucleation of hydroxyapatite crystals	Mimicking bone mineralization with collagen-like fibril structure and nucleation of hydroxyapatite crystals	Promoted new bone formation in a rat femoral defect model	[21]
Cartilage	Peptide amphiphilic nanofibers functionalized with chemical groups of GAG molecules	Mimicking composition, structure and function of the ECM	Enhanced aggregation of MSCs and deposition of cartilage-specific matrix elements	[22]
	Self-assembled supramolecular GAGs like glycopeptide nanofibers	Mimicking composition and functions of HA, the major component of cartilage	Induced chondrogenic differentiation of MSCs and enhanced formation of hyaline-like cartilage	[23]
Heart	Nanofibrous collagen scaffold made by electrospinning and crosslinking for cardiac tissue regeneration	Mimicking composition of myocardial connective stroma and delivery of cardiomyocytes	Improved vascularization of scaffold with upregulation of gene expression related to ECM remodeling, after implanted *in vivo*	[24]
	MSC seeded polycaprolactone nanofiber cardiac patch by fibronectin immobilization	Mimicking ECM of heart by using fibronectin, which is a major component of normal heart for cell adhesion and activity	Enhanced cellular adhesion increased angiogenesis, and improved cardiac function	[25]
Skin	Multilayer nanofilm composed of HA and poly-L-lysine on top of a HA scaffold by using layer-by-layer assembly for skin tissue engineering	Mimicking epidermal–dermal composition and structure of skin at nanometer scale	Promoted adhesion of keratinocytes, enhancing epidermal protective barrier function of skin	[26]
Muscle	Laminin mimetic peptide nanofibrous network	Mimicking composition and structure of skeletal muscle basal lamina	Enhanced cellular gene expression related to skeletal muscle specific marker	[27]

ECM: Extracellular matrix; GAG: Glycosaminoglycan; HA: Hyaluronic acid; MSC: Mesenchymal stem cell.

Nanopatterns play an important role in directing various cellular behaviors, due to their structural consistency with many vital components of native ECM, such as basement membrane and focal adhesion complexes, ranging from a few to a hundred nanometers [48,49]. Patterning techniques at the nanoscale allow for the mimicking of native ECM, thus modulating cell-matrix interactions [50]. Interestingly, nanoscale disorders can direct osteogenic differentiation of human MSCs in the absence of osteogenic supplements [40]. On the other hand, when the pattern contains absolute square lattice symmetry, nanoscale patterning can also promote the growth of stem cells and the retention of multipotency, indicating that nanoscale surface topographies can determine cell fate and functions [41]. Likewise, since cell orientation strongly correlates with the direction of underneath patterns, nanoscale structural cues can further control the macroscopic function of tissue constructs. For example, nanotopographically controlled heart tissue constructs that mimic the ECM structure of myocardium have successfully demonstrated anisotropic action potential conduction and contractility characteristics of native cardiac tissue [39].

Nanopores/channels in natural tissues are also vital for maintaining the activity of cells, as they provide transport paths for oxygen and nutrients [51,52]. While it seems that the two concepts of permeability

Table 2. Examples of mimicking nanoscale tissue structure for tissue regeneration.

Nanostructure	Tissue	Nanotechnology	Tissue regeneration capacity	Ref.
Nanofibrous structures	Heart	Electrospun aligned poly(lactide)- and poly(glycolide)-based scaffold	Demonstrated directionally dependent mature contractile machinery of cardiomyocytes and increased their synchronized beating	[31]
		Highly aligned nanofiber engineered by rotary jet spinning	Induced alignment of rat ventricular myocytes along with the nanofiber	[32,33]
	Tendon	Electrospun aligned PLLA nanofibers	Upregulated tendon-specific genes	[34]
	Cartilage	Nanofibrous hollow microspheres with ECM mimetic architecture as an injectable cell carrier	Induced successful cartilage regeneration in a critical-size osteochondral defect in a rabbit model	[35]
	Skin	3D Multilayered nanofibrous scaffold	Produced dermal-like tissues or bilayer skin tissues with both epidermal and dermal layers	[36]
Nanocomposite structures	Bone	Nanocomposite made from poly(ethylene oxide) and silicate nanoparticles	Induced direction-dependent mechanical properties with increased mechanical strength and extensibility, enhancing cellular activities and mineralization	[37,38]
Nanotopographies	Heart	Myocardium model with controlled nanoscale surface topographies mimicking function of mayocardial tissue and ECM architecture	Displayed anisotropic action potential conduction and contraction of native cardiac tissues	[39]
	Bone	Nanostructured surfaces with symmetry or disorder to modulate stem cell differentiation	Enabled to control MSCs to maintain multipotency or to produce bone minerals depending on nanopatterns	[40,41]
Nanoporous/ nanochannel structures	Bone	Self-assembled hierarchical nanochannel network in bone ceramic	Provided both sufficient mechanical strength and efficient nutrient supply for bone cell growth and differentiation	[42]
	Vessel	Nanopores in the vessel wall mimicking a vascular bed	Enhanced permeability and intercellular crosstalk	[4]

ECM: Extracellular matrix; MSC: Mesenchymal stem cell; PLLA: Poly(L-lactic acid).

and mechanical strength are contradictory, as they are directly or inversely correlated with the porosity of the structures, nanoporous/channel structures can simultaneously satisfy these properties due to their enhanced permeability compared with microporous/channel structures. In fact, the amount of nutrients that are delivered by nanochannels is known to be sufficient to sustain cellular vital activities. Nanopores/channels have been incorporated in vascularized cardiac or hepatic tissue constructs and bone scaffolds by using self-assembled and porogen methods to enhance permeability and permit cellular crosstalk, while maintaining mechanical properties [4,42].

Developing bioinspired nanotechnologies & functional nanomaterials

The function of human tissue occurs based on the localized microenvironment where cells interact with specific types of ECM at the nanoscale. In this respect, nanoscale delivery systems and functional nanomaterials have been applied for directing cellular differentiation and tissue specific activities to restore function of damaged tissues.

In the past two decades, nanoscale delivery systems have attracted a great deal of attention by researchers in the field of regenerative medicine based on their unique features, such as high surface area and easiness of surface functionalization, which can promote the adsorption of growth factors and drugs [53,54]. For example, nanofibers are one of the most widely used nanoscale delivery platforms based on their similarity with the physical structure of ECM [55,56]. Hartgerink *et al.* developed an injectable, self-assembled peptide-based nanofibrous hydrogel that contains peptides for pro-angiogenic moieties which can rapidly form mature vascular networks and induce tissue integration after subcutaneous delivery *in vivo* via a syringe needle [56].

Functional nanomaterials can actively support damaged tissues with functional loss, and thus can enhance their regeneration. For example, electroconductive

Table 3. Developing bioinspired nanotechnologies and functional nanomaterials for tissue regeneration.

Tissue	Nanotechnologies	Functionality	Tissue regeneration capacity	Ref.
Bone	Biomimetic ECM nanostructures constructed through layer-by-layer self-assembly of biodegradable nanoparticles and polysaccharides	Preservation of the activity of osteoinductive growth factors and induced their sustained release	Promoted the attachment, proliferation and differentiation of BMSCs and enhanced new bone formation by sustained release of biomolecules	[60]
	Intermediate precursors-loaded mesoporous silica nanoparticles as delivery devices for biomineralization	Sustained release of amorphous calcium phosphate precursors	Induced biomimetic intrafibrillar mineralization of collagen	[61]
Cartilage	ECM mimetic chondroitin sulfate/polyethylene glycol/GO hybrid nanocomposite scaffold for cartilage engineering	Improvement of overall mechanical properties and electrical conductivity of scaffold by GO	Enhanced regeneration of cartilage tissue with improved subchondral bone reconstruction	[62]
	Bioprinted nanoliter droplets encapsulating stem cells and growth factors to mimic native fibrocartilage microenvironment	Mimicking the complex anisotropic fibrocartilage tissue by 3D printing nanoliter droplets encapsulating MSCs along with biochemical gradient and ECM components	Upregulated osteogenic and chondrogenic related genes in the 3D fibrocartilage model	[63]
	Self-assembled supramolecular peptide amphiphile nanofibers containing binding epitopes to TGF-β-1 for cartilage regeneration	Prolonged release of TGF-β-1 from PA gels containing high density of TGFβ-1 binding sites	Promoted articular cartilage regeneration in a rabbit chondral defect model without any exogenous growth factor	[64]
Vessels	VEGF-loaded heparin-functionalized PLGA nanoparticle–fibrin gel complex	Localized and sustained delivery of growth factor	Improved the therapeutic angiogenic effect in an ischemic hind limb model by increasing blood pressure, angiographic score and the capillary density	[65]
	Biodegradable porous silicon nanoneedles for local intracellular delivery of nucleic acids to induce tissue neovascularization	Codelivery of DNA and siRNA into cell cytosol by nano-injection	Induced localized neovascularization and increased blood perfusion *in vivo*	[66]
	Peptide amphiphile nanostructures that display VEGF mimetic peptide on the surface of nanofibers	Mimicking the activity of VEGF by generating phosphorylation of VEGF receptors	Enhanced proangiogenic activities of endothelial cells and microcirculatory angiogenesis in the ischemic tissue	[67]
Heart	Pluripotent stem cell-derived cardiomyocyte spheroids that incorporate electrically conductive silicon nanowires	Formation of electrically conductive microenvironment in cardiac spheroids which can synergize with exogenous electrical stimulation	Enhanced cell–cell junction formation, increased contractile machinery expression, while regulating the endogenous spontaneous beating of pluripotent stem-cell-derived cardiac spheroids	[57]
	Hybrid hydrogel scaffold incorporating aligned carbon nanotubes	Tunable and anisotropic mechanical and electrical characteristics	Enhanced cardiac differentiation of embryoid bodies with increased beating activity	[58]
Bladder	PLGA nanoparticle thermosensitive gel scaffold for bladder tissue regeneration	Codelivery of growth factors by a PLGA nanoparticle carrier	Promoted bladder tissue regeneration with rapid vascularization while inhibiting graft contracture in a rabbit model	[9]
Nerves	PLGA nanoparticles including LIF as a cargo with surface modification to target OPCs for myelin repair	Sustained and controlled release of LIF by PLGA nanoparticles after selectively attached to OPCs	Induced remyelination with increased myelinated axon numbers and myelin thickness per axon	[68]

BMSC: Bone marrow stem cell; ECM: Extracellular matrix; GO: Graphene oxide; MSC: Mesenchymal stem cell; OPC: Oligodendrocyte precursor cell; PA: Peptide amphiphile; PLGA: Poly(D,L-lactic-co-glycolic acid).

Table 4. Selective list of FDA approved nanotechnology products for tissue regeneration.

Name/company	Approved applications	Product description	Function and clinical outcomes	US FDA approval year	Ref.
Vitoss® scaffold synthetic cancellous bone void filler/Stryker Corporation	Filler, osseous defects	Highly porous 3D β-tricalcium phosphate scaffold based on calcium phosphate nanoparticles	This filler has similar composition to natural bone minerals, enhancing bone regeneration, along with increased spinal fusion rates	2003	[70–72]
Ostim® bone grafting material/Heraeus Kulzer, Inc.	Filler, osseous defects	Nanocrystalline hydroxyapatite paste that is injected into a bone void or defect	This filler facilitates bone regeneration, based on its bone mimetic chemical composition and crystalline structures	2004	[70,73]
NanOss™ bone void filler/Angstrom Medica, Inc.	Filler, osseous defects	Osteoconductive, resorbable bone graft that uses calcium phosphate nanocrystals	This dense, nanocrystalline material mimics the microstructure and composition of bone and has strong mechanical properties and osteoconductive effects	2005	[74,75]
BoneGen-TR/BioLok International, Inc.	Filler, oral surgery, periodontics, endodontics, implantology	Calcium sulfate-based nanocomposite	The filler can control timed release of calcium sulfate that supports bone augmentation	2006	[76]
EquivaBone osteoinductive bone graft substitute/ETEX Corporation	Filler, osseous defects	Resorbable, osteoinductive bone graft substitute that is composed of demineralized bone matrix and nanocrystalline hydroxyapatite	This scaffold has osteoconductive effect by providing hydroxyapatite nanocrystalline and osteoinductive growth factors	2009	[77,78]
Beta-BSM injectable bone substitute material/ETEX Corporation	Filler, osseous defects	Synthetic calcium phosphate bone graft material in a nanocrystalline matrix	This filler has osteoconductive properties based on bone mimetic chemical structure	2010	[78]
NanoGen/Orthogen, LLC	Filler, osseous defects	Medical grade calcium sulfate hemihydrate based nanocomposite	This filler is controlled to be degraded over a period of 12 weeks, stimulating bone regeneration	2011	[79]
FortiCore™/Nanovis, Inc.	Implant, spinal fusion procedures	Implant composed of a highly porous titanium scaffold that is integrated with a PEEK-OPTIMA (high-performance, implant-grade polymer) core	This implant has nanotube-enhanced surface which can promote bone regeneration around the implant	2014	[80]
NB3D bone void filler/ Pioneer Surgical Technology, Inc.	Filler, osseous defects	3D construct that is composed of porous hydroxyapatite nanogranules suspended in a porous gelatin-based foam matrix	This filler has interconnected porosity similar to human cancellous bone and also has equivalent crystal size and structure as natural bone, promoting tissue interaction and regeneration	2014	[81]

nanomaterials have been applied for the treatment of cardiac tissues to generate electrical function of these tissues. The incorporation of electrically conductive silicon nanowires in cardiac spheroids can provide an endogenous electrical microenvironment for cardiomyocytes, and synergize with exogenous electrical

stimulation, enhancing cardiac microtissue development [57]. In addition, when carbon nanotubes are integrated into hydrogels and oriented in an aligned manner, the cardiac differentiation of embryoid bodies and their beating activities are enhanced. The incorporation of carbon nanotubes in a hydrogel scaffold has been reported to further enhance the mechanical properties of tissue constructs [58]. The functionalization of biomaterials by the internalization of biological motifs can also control cellular behavior; for instance, Gouveia *et al.* incorporated peptide amphiphile composed of the N-(fluorenyl-9-methoxycarbonyl) (Fmoc) molecule linked to the cell-adhesion Arg–Gly–Asp–Ser (RGDS) motif into biomimetic collagen gels. These functionalized hydrogels promoted attachment and proliferation of human corneal stromal fibroblasts [59].

In Table 3, we have listed representative examples of the current use of nanotechnologies and nanomaterials to enhance tissue regeneration.

FDA approved regenerative medicine products for tissue regeneration based on nanotechnologies

In the 2014 Guidance for Industry entitled "Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology," the US FDA defined nanotechnology products as those which have at least one dimension between 1 and 100 nm in size [69]. The FDA also recognized materials that are as large as 1000 nm as nanomaterials if they can demonstrate similar 'properties or phenomena' as other nanotechnology-based products [69]. During the process of commercialization, a nanotechnology product moves through various developmental phases, starting with the basic concept product and culminating with clinical investigations and commercialization. The resulting nanotechnology products can belong to various FDA classifications, such as biologicals, devices, genetics, drugs and others [70].

Based on recent achievements in nanotechnologies for recreating the composition, structure and functions of tissues in a more precise way than ever before, the related nanotechnologies are starting to be applied in clinics to repair diseased/damaged tissues [2,70]. In Table 4, we have selectively listed nanotechnology based products for tissue regeneration that have obtained approval from FDA and are currently on the market.

Conclusion & future perspective

In this special issue, we selectively highlighted state-of-the-art nanotechnologies that successfully mimic the composition and structure of different tissue types, as well as bio-inspired nanotechnologies and functional nanomaterials for tissue regeneration. Based on recent advances in nanotechnologies and tissue engineering, bioengineered tissues are becoming more similar to natural tissues, thus enabling the partial recovery of damaged/diseased tissues. However, there are still many biological components that are not fully understood or ignored in regenerative medicine due to the

Executive summary

- This paper highlights the key achievements in the nanotechnology field for regenerative medicine to recreate functional biomimetic tissues and organs.

Biomimicking tissue composition at nanoscale

- Every tissue in the body has its own nanoscale composition.
- Controlling nanoscale composition is important as each tissue type has a unique spatial distribution of materials at the nanoscale which then provides different types of niches for cells.

Mimicking nanoscale tissue structure

- Human tissues have complex topographical features at the nanoscale.
- Nanofibrous and nanocomposite structures, nanotopographies and nanoporous/nanochannel structures have been designed and built by utilizing nanotechnologies such as electrospinning, nanolithography, self-assembly, phase separation and sacrificial template method.

Developing bioinspired nanotechnologies & functional nanomaterials

- Nanoscale delivery systems have provided the sustained and controlled release of growth factors for tissue regeneration.
- Functional nanomaterials have successfully generated similar or even better tissue functions to stimulate cells to repair tissues.

US FDA approved clinical products for regenerative medicine based on nanotechnologies

- Recently, FDA approved nanotechnology based regenerative medicine products have started to be actively used in the clinic for tissue regeneration.
- Most of the current nanotechnology based regenerative medicine products are made for bone tissue regeneration.
- We anticipate that the recent achievements in the nanotechnology field will further lead to the development of regenerative medicine products for various tissue types in the near future.

difficulty in their fabrication. Moreover, although many nanomaterials can successfully promote cellular activities *in vitro*, there still exist safety concerns about the use of these nanomaterials, as they can cause systemic side effects by crossing cell barriers in non-targeted organs. In fact, most of the newly developed nanomaterials have not been assessed in large animal models. As a result, except for bone related materials, the majority of the newly developed nanomaterials have not been applied for tissue regeneration in the clinic. These issues can be addressed by thorough physicochemical characterization of nanomaterials and restriction of undesired uptake via functionalization with targeting moieties [82,83]. Based on the understanding of the effectiveness and safety of nanomaterials, proper *in vivo* studies should be continued with selective nanomaterials for the purpose of clinical translation. We envision that the development of nanotechnologies, which is becoming faster than ever before, will overcome current challenges in regenerative medicine to heal diseased/damaged tissues in the near future.

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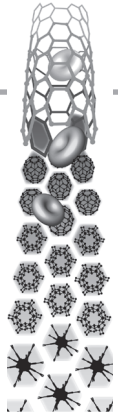

Advanced nanobiomaterial strategies for the development of organized tissue engineering constructs

Nanobiomaterials, a field at the interface of biomaterials and nanotechnologies, when applied to tissue engineering applications, are usually perceived to resemble the cell microenvironment components or as a material strategy to instruct cells and alter cell behaviors. Therefore, they provide a clear understanding of the relationship between nanotechnologies and resulting cellular responses. This review will cover recent advances in nanobiomaterial research for applications in tissue engineering. In particular, recent developments in nanofibrous scaffolds, nanobiomaterial composites, hydrogel systems, laser-fabricated nanostructures and cell-based bioprinting methods to produce scaffolds with nanofeatures for tissue engineering are discussed. As in native niches of cells, where nanofeatures are constantly interacting and influencing cellular behavior, new generations of scaffolds will need to have these features to enable more desirable engineered tissues. Moving forward, tissue engineering will also have to address the issues of complexity and organization in tissues and organs.

KEYWORDS: bioprinting ■ hydrogel ■ laser biofabrication ■ nanobiomaterial ■ nanofiber

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Tissue engineering and regenerative medicine are promising new therapies to meet the global challenge of tissue/organ shortage [1]. However, the philosophy of tissue engineering and regenerative medicine varies considerably with the expertise of individual investigators, some use a biodegradable scaffold while others do not [2]. Currently, the speed of vascularization for implanted engineered tissues is generally low, and viable tissues that can be created, either *in vitro* or *in vivo*, are limited to structurally thin and relatively simple tissues such as skin, cartilage and bladder. Moreover, it is not unusual for the mechanical properties of engineered tissues to be inferior to their native counterparts. It is generally believed that the overall characteristics of an engineered tissue must result from its unique composition and organization of microstructures, such as the organization of cells and extracellular matrices, and that the problem of vascularization and inferiority are likely to be due to the microscale materials and structures within the tissue. Therefore, engineering extracellular matrices and promoting rapid formation of the cellular microenvironment is essential for advancing current tissue engineering and regenerative medicine. The building blocks of extracellular matrices are primarily nano- and micro-scale biomaterials that are dynamically synthesized, organized, remodeled and eliminated by cells. Their temporary presence in tissues usually allows direct physical contact with cell surface receptors, initiating an intracellular cascade of chemical reactions that eventually lead to various phenotypic behaviors such as adhesion, spreading, migration, DNA and protein synthesis, proliferation, senescence, apoptosis, orientation, and alignment. These nano- and micro-scale biomaterials mediate the microenvironment and cellular responses. Correct utilization of these materials can potentially unclock the code of cellular language and instruct cells to release their veiled potential for tissue repair and organ reconstruction.

Nanobiomaterials, at the interface of biomaterials and nanotechnology, refer to a special class of biomaterials with constituent or surface sizes less than 100 nm [3]. Their fine structure allows direct mechanical interactions with cell surface receptors and cellular components, and hence manipulation of cells to serve intended diagnostic or therapeutic purposes. Particularly when applied to tissue engineering and regenerative medicine, nanobiomaterials are usually perceived as microenvironment-like substances in which rich extracellular matrices and various cell types, including stem cells, reside. Nanobiomaterials, when applied to tissue engineering, are usually perceived as having a close resemblance to the microenvironment where cells reside. Through their interaction with cells, nanomaterials act as a means of providing instructive signals to the internal architecture of a cell.

There have been a number of attempts to engineer 3D tissues, but little progress has been made

Future Medicine part of fsg

on engineering 3D organized tissues (FIGURE 1). It is generally believed that the third dimension of an engineered tissue can not exist alone for a long period of time if there is no order being created at the nano- or micro-scale within the tissue. Therefore, it is of paramount importance to acquire further knowledge of nanobiomaterials in order to bridge the gap between biomaterials and nanotechnology, and to reveal their full potential for tissue engineering and regenerative medicine. This review discusses some recent progress on nanobiomaterial strategies in the field of tissue engineering and regenerative medicine, focusing on those with the potential for developing 3D organized tissue engineering constructs.

3D nanofibrous scaffolds & nanocomposites

Nanomaterials are widely utilized in tissue engineering and regenerative medicine because they are able to mimic compositions [4], topographies [5] and architectures [6] of human tissues, and may offer enhanced or new properties to artificial constructs [7]. They have been fabricated into various basic structural units, such as nanoparticles, nanocrystals, nanofibers and nanofilms, to fulfil the specific requirements of biological substitutes that repair or replace malfunctioning tissues [8]. Nanofibrous scaffolds, especially 3D scaffolds, have attracted considerable attention in tissue regeneration in recent years, mainly due to their structural similarity to native extracellular matrix, applicability to a wide range of materials, and readily tunable fiber size and spatial arrangement [9]. To date, nanofibrous scaffolds have been applied in research and regeneration of various tissues (e.g., skin [10,11], vascular [12], bone [13,14], cartilage [15], bladder [16], neural [17,18] and cardiac tissues [19]) *in vitro* and, more significantly, *in vivo*. 2D mats and 3D cotton-like balls are the two typical configurations used for nanofibrous scaffolds. In a recent study, Hsiao *et al.* fabricated an aligned 2D conductive nanofibrous mesh with poly(lactic-*co*-glycolic acid) and polyaniline to induce elongated and aligned rat cardiomyocyte clusters with synchronous cell beating [20]. However, 2D mats are less favorable unless the orientation or functionality of nanofibers is of great importance [21,22]. 2D mats, especially those that are electrospun, usually have a flat topography and tightly packed fibers that restrict cell infiltration to the superficial layers of the scaffolds and cellular integration with host tissue after implantation. Cell sheet technology transforms 2D nanofibrous mats into 3D functional tissues by stacking individual 2D confluent cell sheets recovered from thermoresponsive culture substrates [23,24]. In this manner, it extends the

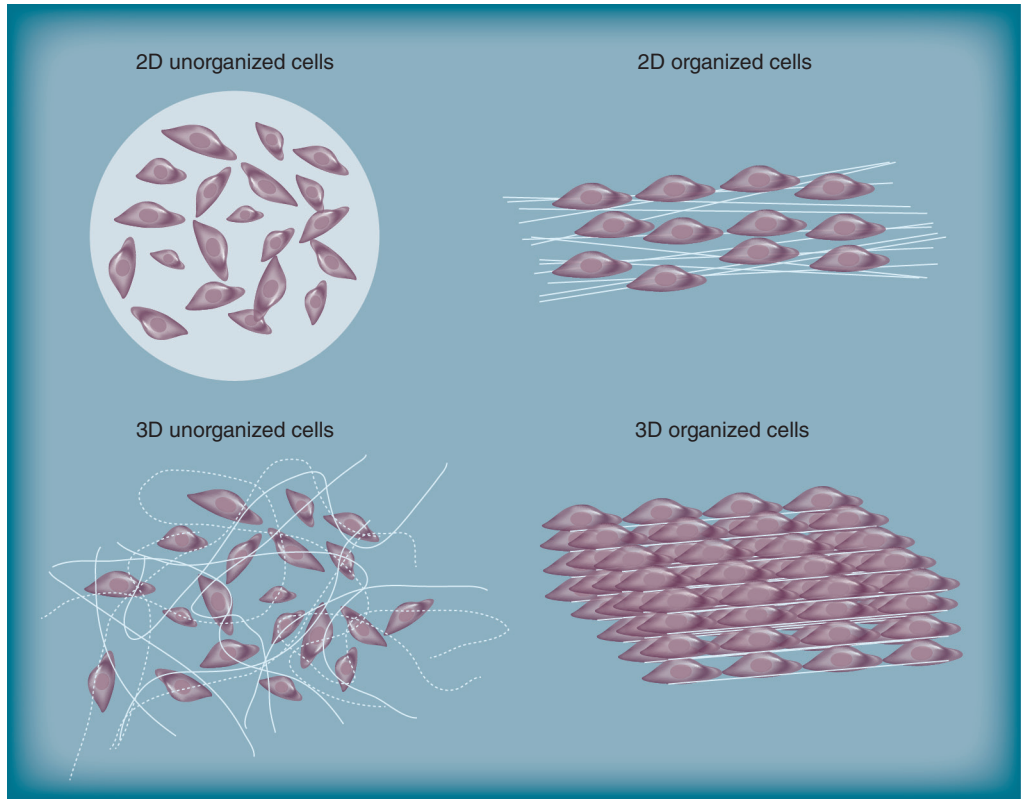

Figure 1. Organization of cells in engineered tissues.

application of 2D mats into producing implantable 3D tissues [25], but its potential will not be fully exploited until good nutrient transportation is achieved in thick cell stacks. Therefore, 3D nanofibrous scaffolds with proper pore size and interconnectivity are highly desirable to enable satisfactory cell infiltration and nutrient diffusion. Electrospinning is the most commonly used technique to fabricate 3D nanofibrous scaffolds with uniform morphology and stability; some are coupled with micrometer-sized framework [26,27] and some are directly electrospun [28,29]. Blakeney *et al.* devised a novel electrospinning collector that is an array of metal probes radially arranged in a spherical foam dish to harvest cotton ball-like poly(ε-caprolactone) scaffolds between the metal probes in mid-air (FIGURE 2). The resulting poly(ε-caprolactone) scaffolds were highly porous and cell infiltration was significantly improved [28]. In another study, Bonino *et al.* reported that 3D alginate nanofiber mats can be electrospun via charge repulsions from negatively charged ions dissociated by the carboxylic acid groups of alginate [29]. In addition to the methods mentioned above, a number of post-electrospinning techniques, such as polymer/salt leaching and laser/UV irradiation, are harnessed to improve the porosity of as-spun scaffolds [30]. Moreover, surface functionalization of electrospun fibers and drug encapsulation with nanofibers can further tailor the nanofibrous scaffolds to improve their performance, for example, by facilitating cell adhesion, spreading and growth, and controlled release of drugs [31].

In order to enhance certain properties or create new functionalities, multicomponent materials may be used to fabricate scaffolds [32]. On top of functional polymers such as electrically conductive polymers, hydroxyapatite, metal nanoparticles and carbon nanomaterials (e.g., fullerenes, carbon nanotubes and graphene) are often incorporated into polymeric matrix to fabricate nanocomposites for applications of tissue engineering and regenerative medicine. Hydroxyapatite, a biocompatible ceramic material mainly used in bone tissue engineering, is capable of resembling bone minerals in morphology and composition [33] and, thus, is extensively employed as part of nanocomposites in bone tissue engineering [34,35]. Electrically conductive materials are usually doped into a polymeric matrix to make conductive fibers/films for stimulating neurons and, hence, neural tissue repair [21]. In a recent study, aligned carbon nanotubes, rolled-up graphene sheets with excellent mechanical and electrical properties, were coated with para-toluene sulfonic acid-doped polypyrrole to form a novel nanostructured conductive platform, in which carbon nanotubes provided the topography and para-toluene sulfonic acid-doped polypyrrole provided the biocompatibility. It has been reported that the rate of differentiation and cell division of primary myoblasts cultivated on the conductive nanocomposite films can be controlled by electrical stimulation [36]. However, the potential toxicity of carbon nanomaterials has always been emphasized [37,38] and, although many *in vitro* experiments have demonstrated that they are nontoxic, the scientific community has to be fully convinced before any significant clinical applications can be realized [39,40]. Metals possess unique physical, chemical and biological properties when downsized to the nanometer scale compared with their macroscopic states. For instance, silver nanoparticles have been used for antibacterial applications. Agarwal *et al.* precisely controlled the loading of silver nanoparticles in thin polymeric films to allow antimicrobial activity without inducing cytotoxicity in mammalian cells [41]. Similar to carbon nanomaterials, the potential risk of metal nanoparticles should be fully understood and controlled before they are utilized in clinical applications.

Hydrogels

Hydrogels consist of a network of crosslinked polymer chains with the ability to absorb large amounts of water without disintegrating. This makes hydrogels unique and attractive as nanobiomaterials for tissue engineering and drug delivery applications. Hydrogels, including thermoresponsive and pH-sensitive gels, have

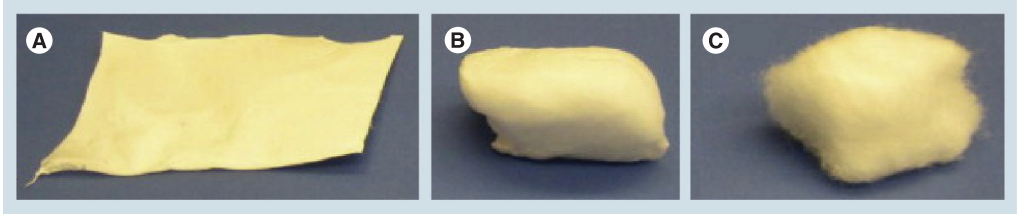

Figure 2. Electrospun nanofibrous scaffolds. (A) 2D nanofibrous scaffolds and **(B & C)** 3D nanofibrous scaffolds.

been researched extensively. Some of the more recent advancement in hydrogels for engineering 3D organized tissues will be reviewed. TABLE 1 shows desired properties of a hydrogel scaffold for 3D organized tissues and currently available strategies to achieve them.

In recent years, bioresponsive hydrogels have progressed substantially, bringing the engineering of a 3D organized tissue a step closer. One of these developments is spatially bioactive hydrogen. In work by Zhu *et al.*, a biomimetic hydrogel scaffold with controlled spatial organization of nanobiomaterials, such as cell-adhesive ligands, was developed [42]. Cyclic Arg–Gly–Asp peptides were first attached in the middle of poly(ethylene glycol) diacrylate (PEGDA) chains and hydrogel formation was initiated via photopolymerization. The authors showed that cyclic Arg–Gly–Asp–PEGDA hydrogels could facilitate endothelial cell adhesion and spreading, and exhibited significantly higher endothelial cell proliferation compared with linear Arg–Gly–Asp-modified hydrogels at low peptide incorporations. Incorporation of cell-adhesive ligands and controlling ligand density and spatial organization is an initial but critical step for hydrogels to be three-dimensionally responsive to cellular adhesions. Stimulation of microenvironmental factors, such as electrical signals, has also been shown to be important because some tissues, such as muscles, require electrical stimuli to function. Mawad *et al.* developed a single component, conducting hydrogel by covalently crosslinking a poly(3-thiopheneacetic acid) hydrogel with 1.1′-carbonyldiimidazole [43]. In addition to swelling ratios up to 850%, the hydrogels were shown to be electroactive and conductive at physiological pH. In terms of cellular responses, fibroblast and myoblast cells were able to adhere and proliferate well on the hydrogel substrate.

One of the common problems with hydrogels is their poor mechanical properties. To enhance the mechanical properties of hydrogels, incorporating nanobiomaterials into hydrogels could be a possible solution. As shown by Kai *et al.*, incorporation of poly(ε-caprolactone) nanofibers into gelatin hydrogel resulted in the increase of the Young's modulus of the composite hydrogels from 3.29 to 20.3 kPa. [44]. Wu *et al.* studied the photocrosslinking of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) triblock copolymer diacrylates (Pluronic® F127 diacrylate; BASF, Ludwigshafen, Germany) in the presence of the silicate nanoparticle Laponite® (Rockwood Additives, TX, USA) and the resulting hydrogels had high elongations and improved toughness [45]. Chang *et al.* developed PEGDA/Laponite nanocomposite hydrogels, and the incorporation of Laponite nanoparticles significantly enhanced both the compressive and tensile properties of PEGDA hydrogels [46]. The authors also demonstrated that their nanocomposite hydrogels were able to support 3D cell culture. In addition to mechanical advantages, incorporation of nanobiomaterials also offers bioactive advantages. Azami *et al.* prepared a gelatin–amorphous calcium phosphate nanocomposite scaffold that has a three-dimensionally interconnected porous microstructure. After incubation in simulated body fluid solution at 37°C for 5 days, the mineral phase of the scaffold was transformed into nanocrystalline hydroxyapatite [47]. Sowmyaa *et al.* reported a chitin hydrogel scaffold lyophilized with bioactive glass ceramic nanoparticles, which was found to have enhanced porosity, swelling, bioactivity and degradation [48]. Moreover, the composite scaffolds were nontoxic to human osteoblasts and suitable for periodontal bone defects. Sudheesh Kumar *et al.* developed chitin/nanosilver composite scaffolds that were effective against *Escherichia coli* and *Staphylococcus aureus* [49].

Spatially controlled release of growth factors is a desired property of a tissue engineering scaffold, and this may be conveniently realized after the invention of nanogels. Nanogels are a special type of hydrogel in which hydrogel nanoparticles or nanogels (<100 nm) are either chemically or physically crosslinked by polymer chains to form a 3D network [50]. Owing to their nanometer size, nanogels are more effective at stably trapping bioactive compounds inside their network and respond more rapidly to microenvironmental factors such as temperature and pH. Therefore, nanogels are important for spatially controlled release of growth factors within a scaffold. FIGURE 3 shows a schematic of the preparation of a cholesterol-bearing pullulan nanogel-crosslinking hydrogel to deliver BMP-2 [51]. Hayashi *et al.* examined the efficiency of

Table 1. Nanobiomaterial strategies for enhancing the properties of hydrogels.

Desired properties of hydrogel scaffold	Nanobiomaterial strategies
Bioresponsiveness	Nanoscale ligands
Mechanical strength	Nanocomposites
Controlled release of growth factors	Nanogels
Properties of native proteins	Self-assembled peptides

nanogels to deliver BMP-2 *in vivo* for bone defect repair. Despite a single implantation with low amounts of BMP, vigorous osteoblastic activation and new bone formation were evident [51]. Kamolratanakul *et al.* went further by delivering a combination of a selective EP4 receptor agonist and a low dose of BMP-2 in a nanogel-based disc scaffold, and observed efficient activation of bone cells and effective regeneration of bone tissues [52]. In another study, Bencherif *et al.* hybridized nanogels to hyaluronic acid by mixing them under physiological conditions (pH = 7.4; 37°C), and created a nanostructured hyaluronic acid hydrogel scaffold with a porous 3D uniform distribution of nanogels [53].

In addition to the aforementioned properties, there is considerable interest in developing self-assembled peptide nanostructure to

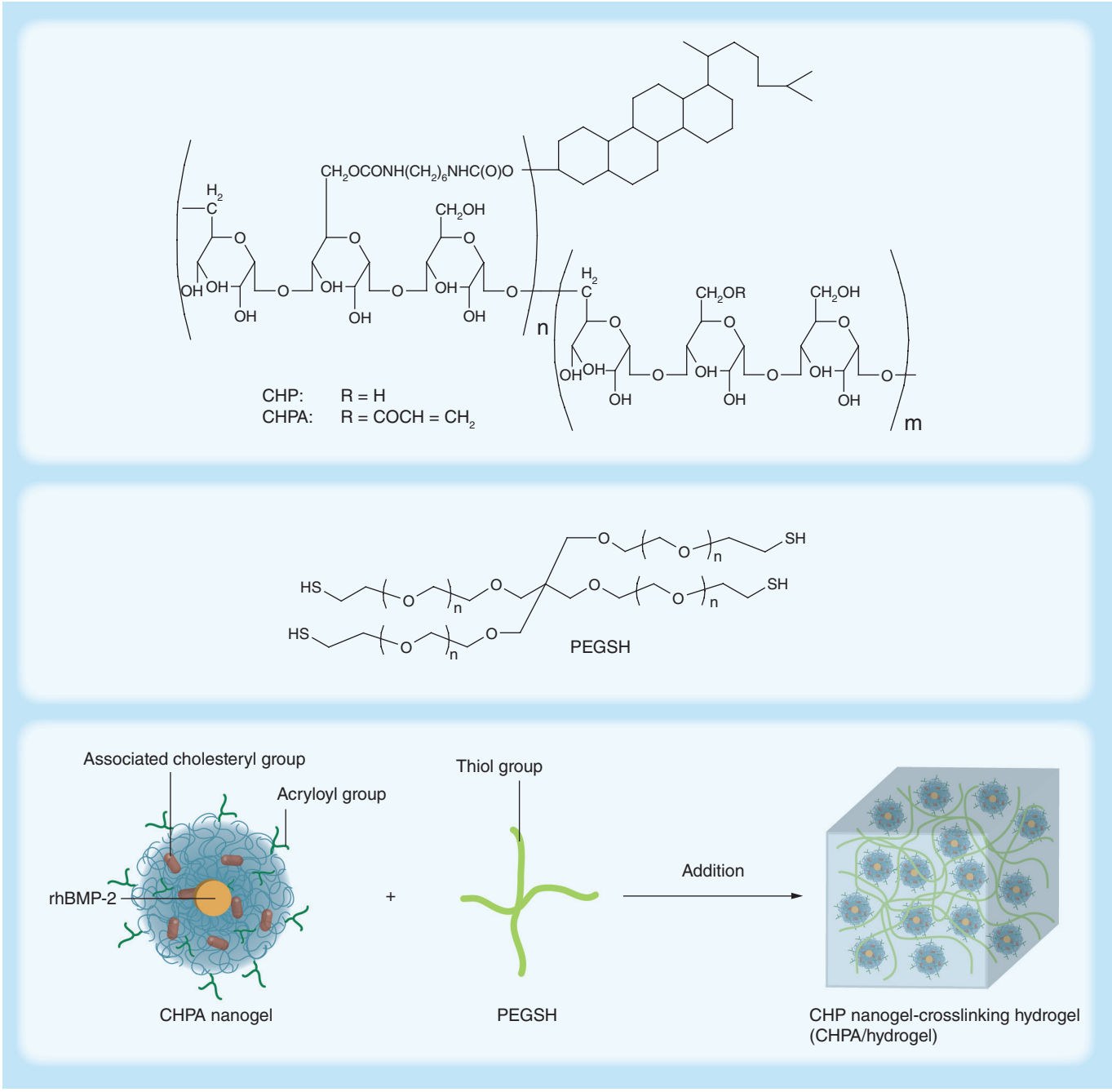

Figure 3. Acryloyl group-modified cholesterol-bearing pullulan nanogel-crosslinking hydrogel containing BMP-2 growth factor.
CHP: Cholesterol-bearing pullulan; CHPA: Acryloyl group-modified CHP; PEGSH: Thiol group-modified poly(ethylene glycol); rhBMP-2: Recominant human BMP-2.
Reproduced with permission from [51].

mimic the creation process of native proteins. O'Leary *et al.* designed a peptide sequence (Pro–Lys–Gly)$_4$(Pro–Hyp–Gly)$_4$(Asp–Hyp–Gly)$_4$ that can form a stable triple helix and replicates the self-assembly of collagen through all steps. The resulting nanofibres can form a hydrogel that is degraded by collagenase at a similar rate to that of natural collagen [54]. The ability to design and synthesize peptides with characteristics that are similar to their native counterparts can offer significant advantages in the control and manipulation of scaffold properties.

Laser-fabricated 3D nanostructures

Laser technology is able to generate fine features, such as ridges, grooves and standing rods, among others, on a 2D surface, and it has shown remarkable influence on various cell behaviors, including cell attachment, orientation, proliferation and differentiation [55–58]. Most, if not all, of these studies are conducted on a 2D platform on which the properties of the nanoscale features such as spacing, width and height of ridges are constructed. The laser forms different features that mimic natural extracellular matrix features, which causes the cells to interact with the artificial construct as they would *in vivo*. Using laser-machined biomaterials with nanoscale features may potentially help us to gain a better understanding of biological mechanisms, such as cell adhesion on a biomaterial surface, which is mainly directed by molecular interactions at the nanoscale [59]. These studies contribute greatly to the fundamental understanding of the role of nanoscale topography, but have little correlation with the role of spatial nanostructures on 3D tissues. One of the main reasons this area is not progressing as fast is the lack of adequate methods to generate 3D nanostructures. Additive manufacturing technology is a group of techniques that could possibly address this, as it is able to fabricate 3D constructs based on a layer-by-layer principle.

The advantages of using the additive manufacturing approach to fabricate 3D nanostructures is the controllability of process parameters and, hence, the resulting consistency of scaffold properties. Selective laser sintering [60,61] and stereolithography [62,63] are two widely used techniques to fabricate 3D scaffolds for tissue engineering and regenerative medicine applications. However, distinct disadvantages limit their application. One of the obvious drawbacks is resolution, or rather the lack of resolution, as selective laser sintering and stereolithography can only fabricate precisely controlled scaffolds with geometrical dimensions ranging from tens to hundreds of micrometers, which is too large to mimic the unique microenvironment of natural tissues *in vivo* with submicron and nanoscale cues. With recent advancements in 3D laser nonlinear lithographic technology [64,65], multiphoton polymerization, especially two-photon polymerization (2PP), has been applied to create 3D nanostructures in a scaffold [66,67]. This technique has achieved the highest resolution (with feature sizes as small as 100 nm and even a size of 30 nm has been reported [68]) so far in the family of additive manufacturing technology. The resolution of 2PP is adjustable, which conveniences the tuning and thus saves fabrication time [69].

2PP has been applied to a wide range of materials, from synthetic polymers (e.g., biodegradable triblock copolymer [70] and nonbiodegradable polymer Ormocer®, VOCO GmbH, Cuxhaven, Germany [71,72]) to proteins (e.g., fibrinogen [73,74], collagen type I and bovine serum albumin [75]), and even different metal-based sol–gel composites (e.g., Zr- or Ti-based composites [76,77]). Among these widely used materials, PEGDA, with its biocompatibility and nonfouling properties, is a very good candidate for tissue engineering scaffold fabrication after 2PP treatment [78,79]. Figure 4 shows a scaffold fabricated by 2PP. The 3D structure is sophisticated and intricate, with a minimum feature size of 200 nm. Many PEGDA-based 3D scaffolds formed by 2PP have already been evaluated for their biocompatibility, including cytotoxicity [78], cell adhesion and cell viability [80]. There is a report that even showed a promising approach through the integration 2PP and laser-induced forward transfer to fabricate arbitrary PEGDA-based 3D structures with predesigned submicron features [81]. This technique offers a new approach to achieve 3D multicellular tissue constructs with an engineered extracellular matrix. It is also very interesting to notice that 2PP can crosslink natural polymers, potentially allowing the exploration of proteins and DNA as templates for the construction of 3D scaffolds [82]. In their report, the authors found that the laser formed protein scaffolds with precisely designed topographies that could be used as a new bioelectronics platform for monitoring and simulating biological processes [82], such as cellular signal transduction and neuronal networking [83,84]. Many properties of 2PP-generated 3D structures can also contribute to medical devices, such as small prosthetics [71,85,86]. Ovsianikov and coworkers manufactured total ossicular replacement prostheses out of Ormocer [87]. 2PP is a very important process in the synthesis

of Ormocer. The flexibility of 2PP makes the dimension of total ossicular replacement prostheses adjustable, which would be conducive to regenerative medicine applications.

Although 2PP has already proven to be a powerful technique for tissue engineering scaffold fabrication, there are still some drawbacks that limit its widespread usage. One of these factors is production time. Recently, several advanced methods have been explored to improve the throughput of laser fabrication. In a study by Zhang and Chen, the combination of 2PP and nanoimprinting was presented as an effective way to produce nanofeatures in the hydrogel in a massively parallel way [67]. Another trial utilized multibeam fabrication to shorten the 2PP process [88].

Using a laser to create nanofeatures is proven for 2D structures, but for 3D structures there remain challenges. However, there are emerging techniques that have shown feasibility, and as science and technology advances, this is probably going to be a very viable method.

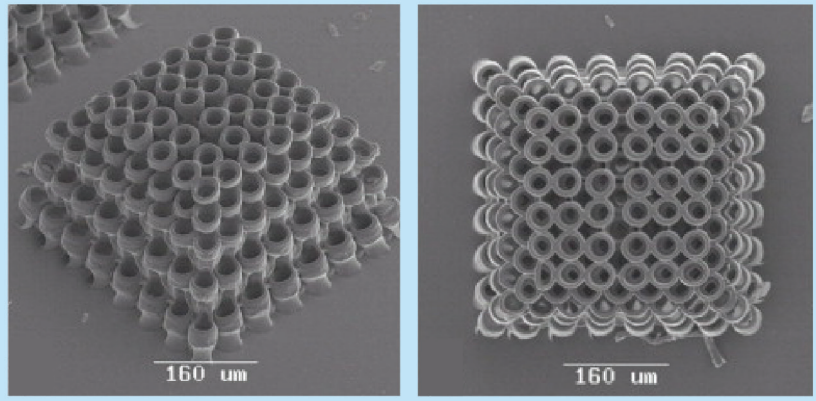

Figure 4. Highly organized 3D scaffold structure fabricated by two-photon polymerization. The minimum feature size is 200 nm.
Reproduced with permission from [78].

Bioprinting of cells

All the techniques discussed above involve the fabrication of advanced scaffolds to support cells.

In this section, another method to directly manipulate the microstructure of tissues at the cellular level to build up the organization of tissue, without the use of scaffolds, will be discussed. Bioprinting refers to a special additive manufacturing technology that processes cells and biological materials into the physical counterpart of a predefined 3D computer model. From the point of view of manufacturing, the resolution of the bioprinting process is below 100 µm, although not within the nanoscale. However, from the point of view of interactions between cells and materials, the scale of bioprinted biologics ranges from micrometers (e.g., cells) to nanometers (e.g., focal adhesion complexes and integrins).

In one study of bioprinting, cells were first prepared in the form of tissue spheroids in a robotic system [89], and then mixed with a hydrogel and printed one-by-one in a defined layout, such as a ring or a branched structure. Over time, these printed tissue spheroids can fuse and integrate to form tissue with an ordered organization [90]. In another method, a laser was used to assist printing of controlled 2D cellular patterns, such as the Olympic symbol shown in FIGURE 5, in a high-resolution and high-speed manner at the microscale [91]. Recently, multiple cell types have been separately mixed with crosslinkers ($CaCl_2$) and loaded into separate ink cartridges for inkjet printing [92]. The multiple-cell pie configuration shown in FIGURE 6A consists of human amniotic fluid-derived stem cells, canine smooth muscle cells and bovine aortic endothelial cells. All printed cell types maintained their viability and normal physiological functions within

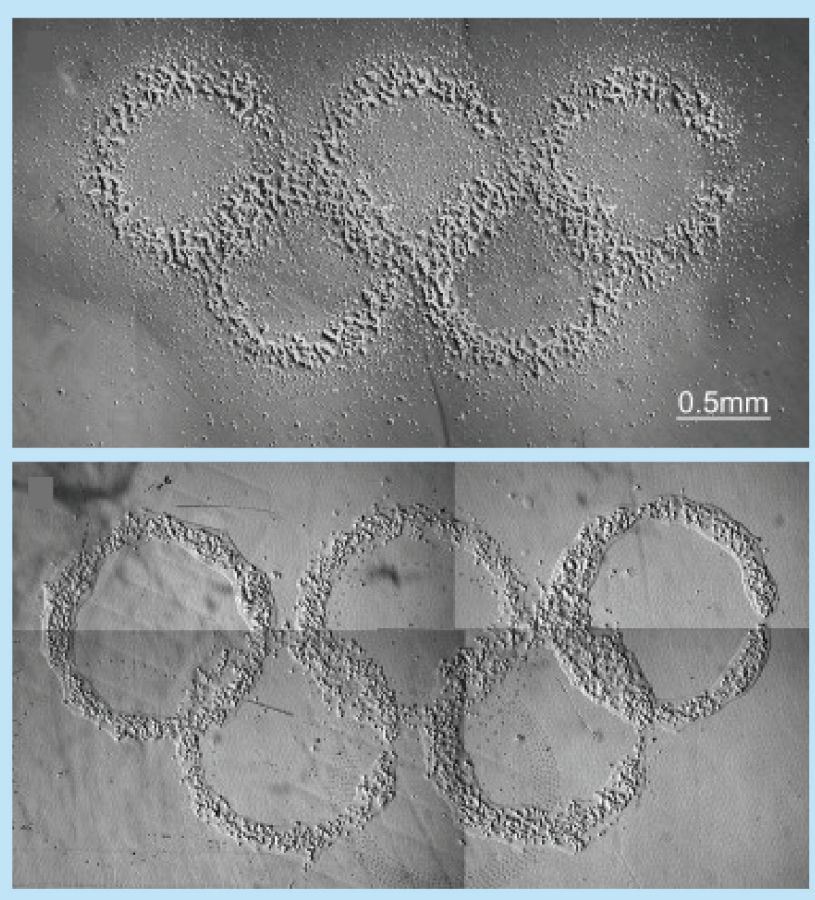

Figure 5. Laser-assisted bioprinting of 2D cellular patterns.
Reproduced with permission from [91].

the hybrid constructs (FIGURE 6B). The bioprinted constructs were adequately vascularized *in vivo* and matured into functional tissues (FIGURE 6C).

Bioprinting of cells is very much in its infancy and there are practical challenges ahead. Currently, one practical limitation of bioprinting is the weak mechanical strength of bioprinted hydrogels [93]. Development of a bioprintable hydrogel that is suitable for the bioprinting process, as well as for cell encapsulation and viability, is critical. Censi *et al.* evaluated the suitability of a biodegradable, photopolymerizable and thermosensitive A–B–A triblock copolymer hydrogel, in which poly(*N*-(2-hydroxypropyl) methacrylamide lactate) forms A blocks and hydrophilic poly(ethylene glycol) forms B blocks [94]. They demonstrated layer-by-layer deposition of hydrogel fibers, forming stable 3D constructs with high viability of encapsulated chondrocytes. Another practical challenge in bioprinting is the concurrent printing and culture of mixed multiple cell types. There are a few approaches that may be considered for fabricating a construct with mixed multiple cell types; for example, deposition of multiple types of cells through multiple nozzles or deposition of tissue spheroids that already contain a mixture of multiple cell types. Nonetheless, these approaches only address the issue of how to aggregate multiple types of cells; at the fundamental level, how to concurrently culture and grow multiple cell types is still unclear. Norotte *et al.* reported the use of various vascular cell types, including smooth muscle cells and fibroblasts, for bioprinting [2], but these cell types were not seeded at precise locations within a single scaffold and post-printing culture has not involved in their study. Although Schuurman *et al.* claimed to be able to bioprint a hybrid tissue construct [95], their actual work is limited to multiple cells of a single cell type, not multiple cell types. Currently, there is also considerable interest in the strategy of *in situ* bioprinting [96], in which the mixture of cells and hydrogels are directly deposited onto defect areas such as skin burns. This strategy could potentially eliminate the problems of *in vitro* bioprinting and provide rapid tissue repair, thus promising to be a new therapy in the future.

Conclusion

In conclusion, various nanobiomaterial strategies have shown some promising aspects in terms of

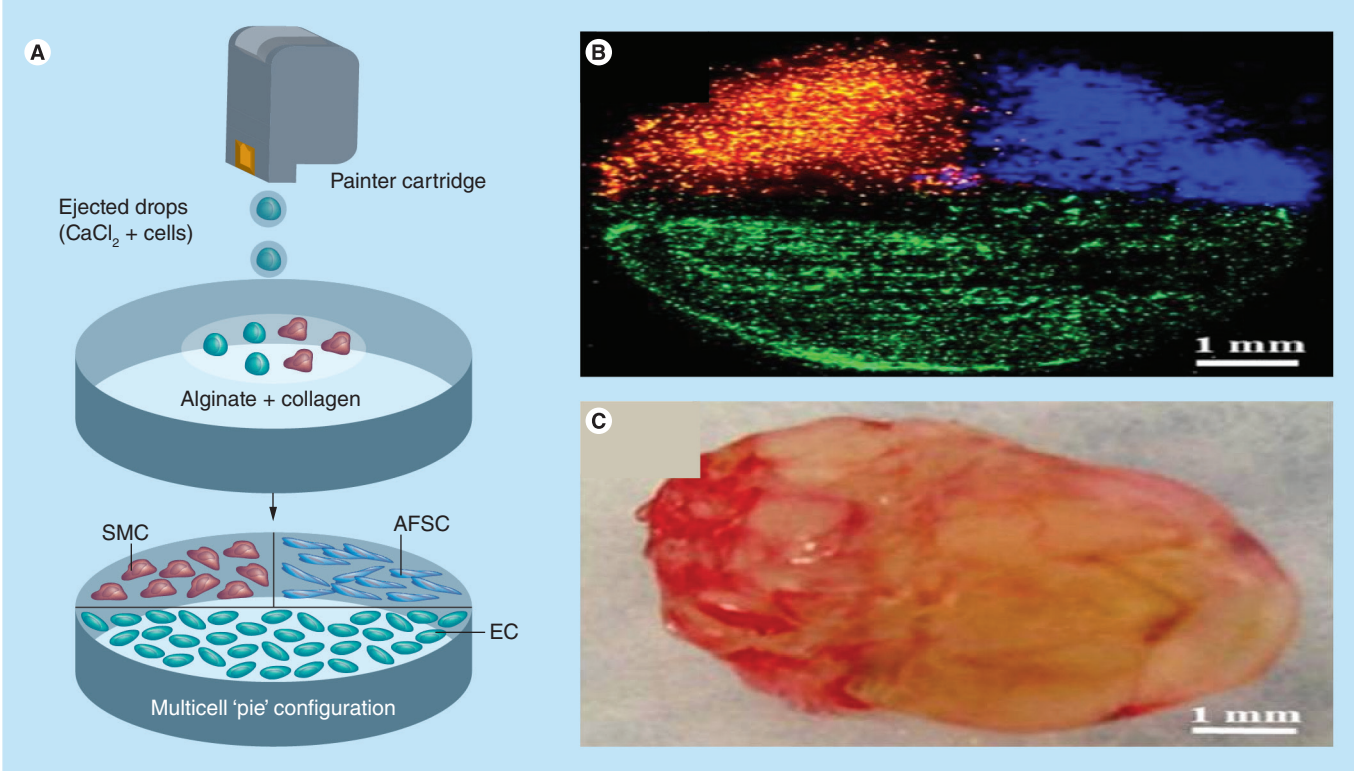

Figure 6. Inkjet bioprinting of 3D tissue engineering constructs consisting of multiple cell types. (A–C) The multiple-cell pie configuration. **(B)** All cell types maintained their viability and normal physiological functions within the hybrid constructs, **(C)** which were adequately vascularized *in vivo* and matured into functional tissues.
AFSC: Amniotic fluid-derived stem cell; EC: Endothelial cell; SMC: Smooth muscle cell.
Reproduced with permission from [92].

Table 2. Nanobiomaterial strategies for developing 3D organized tissue engineering constructs.

Nanobiomaterial strategy	Advantages	Disadvantages	Organization
Nanofibrous scaffolds and nanocomposites	Established 2D nanofeature guidance on cells and 3D bulk nanofibrous constructs available	Unorganized 3D bulk structure, inadequate pore size and strength, and poor consistency	Unorganized
Hydrogels	Ability to hold water and swell, resemble living tissues and ease of applicability	Inadequate bioactivity and strength, and poor internal structure	Less organized cell–scaffold constructs
Laser fabrication	Rapid fabrication and highly controllable organized 3D scaffolds available	Few biomaterials can easily be laser processed	Organized scaffold structure
Bioprinting	Established principle of layer-by-layer printing of preliminary cell/tissue constructs	Inadequate hydrogel strength, and great biological challenge of printing and culture of heterogeneous cells	Organized cells and tissue structure

Nanobiomaterial strategies are listed in ascending degree of organization.

addressing the issue of complexity and organization in tissues and organs, but no single strategy is a complete solution to this challenge. TABLE 2 summarizes the advantages and disadvantages of each and suggests that, based on the degree of organization of current 3D constructs, the bioprinting strategy is now closer to these aims than other strategies, and could be a viable approach in the future.

Future perspective

Various recent nanobiomaterial strategies have been reviewed in this paper to highlight their potential for engineering 3D organized tissue. Moving forward, tissue engineering and regenerative medicine will have to address the issues of complexity and organization in tissues and organs. Future work should include manipulation of nanobiomaterials toward the engineering of a more ordered 3D tissue microstructure, and should reveal more on the relationship between tissue microstructure and the resulting characteristics of an engineered tissue.

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Executive summary

- Tissue engineering and regenerative medicine are restricted by a limited tissue thickness and poorly organized tissue microstructures.
- In recent years, research on nanobiomaterials for tissue engineering and regenerative medicine applications has emerged, due to their ability to direct cell behaviors toward desired tissue outcomes.
- Recent progresses in 3D nanofibrous scaffolds and nanocomposites, hydrogels, laser-fabricated nano- and micro-structures, and bioprinting enable the possibility of developing 3D organized tissue engineering constructs to address the issues of complexity and organization in tissues and organs.
- Bioprinting is a very promising approach for developing 3D organized tissue constructs, but it is still in its infancy and is yet to overcome the practical challenges to truly deliver a printed functional tissue or organ.

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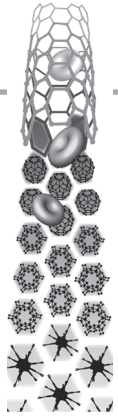

Laser-assisted cell printing: principle, physical parameters versus cell fate and perspectives in tissue engineering

We describe the physical parameters involved in laser-assisted cell printing and present evidence that this technology is coming of age. Finally we discuss how this high-throughput, high-resolution technique may help in reproducing local cell microenvironments, and thereby create functional tissue-engineered 3D constructs.

KEYWORDS: bioprinting ▪ cells ▪ modeling ▪ rapid prototyping ▪ tissue engineering

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On tissue engineering approaches

The loss or failure of an organ or tissue is one of the most frequent, devastating and costly problems in healthcare [1]. Current treatment modalities include transplantation of organs, surgical reconstruction, use of mechanical devices or supplementation of metabolic products. Epidemiological studies highlight tissue/organ shortage, which justifies original approaches to fulfill clinical needs [2]. Tissue engineering (TE) aims at providing regenerative medicine with original products. In addition, TE may provide original models (2D/3D) for fundamental research in biology [3]. Since the late 1980s and the creation of the first workable definition of hybrid artificial organs [4], an increasing number of research groups throughout the world have developed TE-orientated approaches. As stated by Langer and Vacanti [5], these approaches apply the principles of engineering and life sciences to the development of biological substitutes that restore, maintain or improve tissue or whole organ function. Generating biological tissues *in vitro* involves the use of engineering and material methods, the appropriate combination of cells and the suitable biochemical and physicochemical factors to mimic both the microenvironment of cells and the microarchitecture of tissues in the body. Traditionally, TE approaches use porous biomaterial scaffolds seeded with isolated autologous cells from the patient, culturing the constructs in a bioreactor and implanting the resulting cell/biomaterial complex back into the patient. With an appropriate scaffold that mimics the biological extracellular matrix, it is expected that the developing tissue will adopt both the form and function of the desired organ. Tremendous progress in the synthesis and manufacturing of biomaterials in order to obtain highly biocompatible and functional scaffolds has been made.

Tissue engineering has already proven its ability to move from the bench to the bedside [6]. For instance, blood vessel-, skin-, cornea- and bladder-related TE products have been successfully translated from research to clinical practice [7–11].

Despite these scientific progresses and clinical outcomes, engineered tissues and especially thick or complex tissues still suffer from reoccurring drawbacks:

- Cell penetration and adhesion is not very effective. One or several months may be required for the cells to adhere and proliferate into the scaffold. As a result, an incomplete colonization, limited to the scaffold's external layers, may occur [12];
- Organs and tissues are generally complex, and host different cell types. Cell-to-cell contact and cell-to-substrate interaction is critically involved in tissue morphogenesis and regulation, or healing. Consequently the need to promote cell-to-cell communication remains a very challenging issue for this kind of approach;
- The absence of built-in vascularization [12,13]. Most tissues in the body rely on blood vessels to supply individual cells with nutrients and oxygen. For a tissue thickness beyond 100–200 µm (the diffusion limit of oxygen), new blood-vessel formation is required, and this holds true for TE constructs [14].

The limitations of the scaffold-based TE lie in the initial paradigm of creating a functional 3D tissue structure by seeding cells (differentiated or stem cells) onto the scaffold, without any *ab initio* patterns. In other words, the question

future medicine part of fsg

of "what 3D pattern of cells and biomaterials must be fabricated?" has been consistently either eluded to or ignored, probably because no relevant technology has previously existed. This question, which is similar to Rivron's "How to orchestrate developmental mechanisms *in vitro*?" [15] is now a leading concern and should become the focus of studies in the future.

In this report we discuss the need to develop tools dealing with tissue complexity and anisotropy, and physical parameters involved in laser-assisted cell printing. We then present evidence that this technology is coming of age. Finally we discuss how high-throughput, high-resolution techniques may address these questions in the near future.

From macroscopic to microscopic TE

Stem cell fate is influenced by a number of factors and interactions that require robust control for safe and effective regeneration of functional tissue [16]. Coordinated interactions with soluble factors, other cell types and extracellular matrices define a local biochemical and mechanical microenvironment with complex and dynamic regulation that stem cells sense [15]. On a local scale, tissue development is, in part, regulated by the spatial and temporal distribution of cues (i.e., local gradients of soluble or insoluble factors, local physical forces). This suggests a dynamic interaction between form and function [17,18] and emphasizes the importance of shaping adequate multicellular, multifunctional geometries to promote proper tissue integration.

Tissue engineering approaches can be divided into three strategies based on the scale of spatial organization. First, macrosopic strategy can be likened to traditional TE in which cells are seeded onto a macroporous scaffold. Cells are expected to colonize the inner volume of the scaffold by cell mobility and proliferation, and fluid flow. As described above, advances in the design of smart scaffolds and in the understanding of tissue maturation within bioreactor chambers have produced functional tissues [19]. However, smart scaffolds do not present the ability to mimic the functional multicellular anisotropy of the host tissue. Second, mesostructures are based on cells' ability to self-assemble and their capacity to maintain viability and function when located within the diffusion limit of nutrient supply. These modular blocks, also termed organoids, can be fabricated *in vitro* using replica molding [20,21] or by shaping multicellular spheroids [22]. The modular approach enable the production of 3D modules in a variety of shapes (e.g., cylinders) with a lateral diameter between 40 and 1000 µm and cell densities of 10^5–10^8 cells/cm², and to allow fabrication of multicellular constructs (e.g., bone-mimicking construct including both osteoblasts, osteoclasts and endothelial cells). Finally, reproducing the local cell microenvironment can be thought of as the ultimate target for TE and cell patterning. Conceptually, it could be defined as the capacity of positioning a single cell into its most suitable environment. Coordinated interactions between soluble factors, different cell types and extracellular matrices (i.e., mechanical and biochemical cues) should be taken into account. Such a cell niche manufacturing approach is unique in its purpose of dealing with tissue complexity and engineering a desired tissue from the bottom up. A scaffold-free, bottom-up approach to TE has been proposed [23]. Interestingly, Albrecht *et al.* have presented a method for the rapid formation of reproducible, high-resolution 3D cellular structures within a photopolymerizable hydrogel using dielectrophoretic forces [24]. This technology allows production of microscale cell organization. However, deposition of extracellular material can not be geometrically controlled using this approach.

To address some of the aforementioned issues, some authors have suggested building 3D biological structures using bioprinting: the precise computer-aided robotic deposit of living and nonliving biomaterials with the purpose of bioengineering 2D cellular patterns and 3D tissue constructs [25]. Commercially available ink-jet printers have been successfully employed [26,27] to pattern biological assemblies according to a computer-aided design template (blueprint). Pressure-operated mechanical extruders have also been developed to handle cells and cell aggregates [28]. Laser-assisted printing has emerged as an alternative technology, which has the ability to overcome some of the limitations of ink-jet and micro-pen printing devices, namely, the clogging (due to viscosity, cell agglomeration or ink drying) of print heads or capillaries used by these printers to achieve micron-scale resolution. In addition to laser-guided direct writing, which is a technique capable of trapping multiple cells in a laser beam and depositing them as a steady stream on arbitrary nonabsorbing surfaces [29], laser-assisted bioprinting (LAB) has been developed. Based on the laser-induced forward transfer (LIFT) process, LAB is potentially more efficient than laser-guided direct writing for cell patterning and TE; optical trapping allows manipulation of individual cells or cell aggregates in a matter of seconds, while LAB can manage virtually single cell deposit at a rate of kHz.

Mechanism of laser-assisted bioprinting

A typical LAB set-up is generally composed of three elements: a pulsed laser source, a target coated with the material to be printed (the ribbon) and a receiving substrate (Figure 1). The ribbon is a multilayer component: a support, which is transparent to the laser radiation wavelength, is coated with a transfer layer, named bioink, composed of the heat sensitive biological material to be printed (e.g., biomaterials, cells, biomolecules). Depending on the optical properties of the bioink or laser wavelength, a laser-absorbing interlayer is necessary to induce transfer and is placed between the support and the bioink; hence, the term biological laser printing [30] or absorbing-film assisted-LIFT [31] is preferred to matrix-assisted pulse laser evaporation-direct write (MAPLE-DW) [32], which implies the vaporization of the first molecular layers of the liquid. Such an interlayer eliminates direct interaction between the laser beam and the bioink, while 99% of the nonreflected incident beam may be transmitted in the case of MAPLE-DW. This interlayer consists of a thin film (tens of nm) of metal (Au, Ti, Ag), metal oxide (TiO_2) or photo-decomposing volatile polymer (triazene). Even if the LAB printing mechanism depends on many parameters (Figure 1), it was found that the volume of deposited material depends linearly on the laser pulse energy, and that a minimum threshold energy has to be overcome for microdroplet ejection to occur [33,34]. The technology behind these techniques has been increasingly refined in recent years giving new highlights to the three regimes which are typically conserved experimentally: subthreshold, jetting and plume regimes [35]. LAB can be described by the following sequence of events.

Laser energy deposit (1 J/cm²)

Pulsed-energy deposition (typically 1–20 µJ per pulse) can be performed by means of nano second lasers with UV wavelengths (i.e., excimer lasers 193 nm, 248 nm or triple- or quadruple-frequency neodymium-doped yttrium aluminium garnet lasers (266 and 355 nm, respectively) or else with near IR wavelength (1064 nm). The laser energy-absorbing material at the bioink–support interface (as in MAPLE-DW) or of/near the absorbing interlayer (as in biological laser printer) rapidly evaporates (including normal boiling and phase explosion) upon the absorption of laser pulse energy and further plasma formation, and may further form a vapor bubble that expands towards the free surface.

Vapor bubble growth & collapsing (1 µs)

Bubble growth and collapsing are critical phenomena since they govern the printing regime and because they are related to the intensity of mechanical stress applied to cells within the bioink (see below). In addition to time-resolved imaging, which provided time scale and morphological information [35,36], bubble dynamics have been modeled analytically (using the Rayleigh–Plesset equation) and

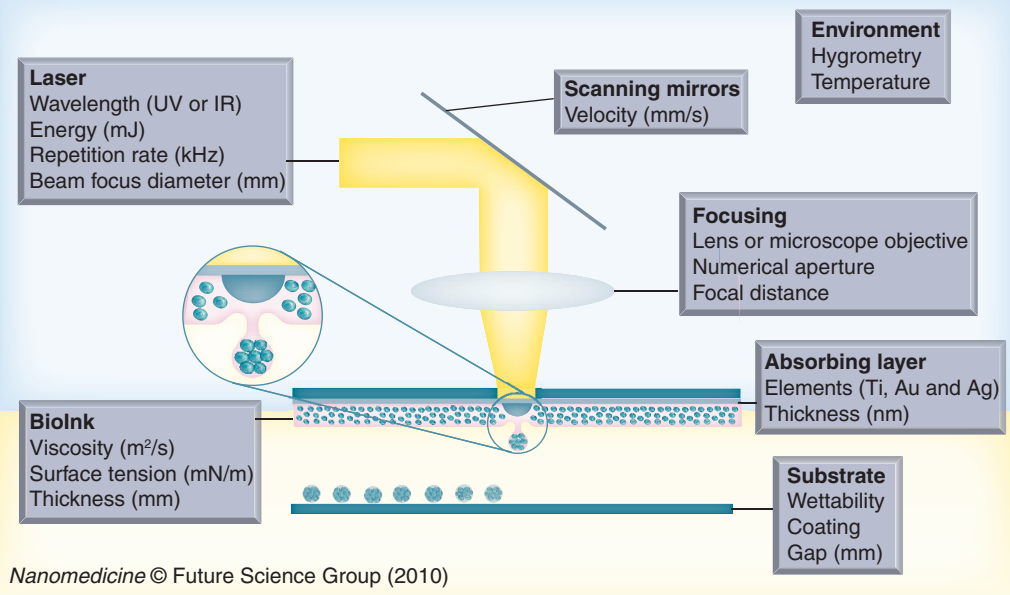


Figure 1. Laser-assisted bioprinting, featuring all the parameters involved in the process.

numerically [37,38] by including the effect of different medium material properties (e.g., viscosity and surface tension).

$$RR+\frac{3}{2}R^2=\frac{\kappa P\iota}{\rho\iota}\left(\frac{R_0}{R}\right)^{3y} - \frac{P\iota - P\upsilon}{\rho\iota} - 4\upsilon\frac{R}{R} - \frac{2\sigma\iota}{\rho\iota R}$$

As a result, it was demonstrated that, depending on the bioink compressibility, the bubble front rapidly reaches its maximum velocity (up to 100 m/s, 100 ns after plasma-induced generation) while the maximum bubble radius R_{max} (meaning when collapsing starts) was reached later (1.2 µs) [34]. These values were shown to diminish by increasing medium viscosity while bubble dynamics was shown to be insensitive to surface tension.

Interaction of the vapor bubble with the free surface

Since the size of the vapor bubble is negligible compared with bioink thickness, the bubble interacts with the free surface, and hence surface tension has to be taken into account. In this regard, it has been demonstrated (for standoff conditions) that when the bubble reaches R_{max}, it begins to collapse due to a high pressure region generated in the bubble apex, and a jet may be formed according to the dimensionless distance Γ, which is the ratio between the distance, h (distance between the initial vapor bubble centroid and the free surface), and R_{max} [39,40].

$$\Gamma = \frac{h}{R_{\max}}$$

Consequently, the three above-mentioned regimes do not solely result from laser energy (E) intensity but also from rheological properties (e.g., viscosity [υ], surface tension [σ]) and film thickness (ε) of the bioink. In other words, jetting does not simply occur on the basis of an energy threshold mechanism [33] but rather on the basis of a complex Γ (E,υ,ε,σ) threshold mechanism. Over a given laser energy for which a vapor bubble is formed at the absorbing layer–bioink interface, the three above-mentioned regimes can be described (Figure 2):

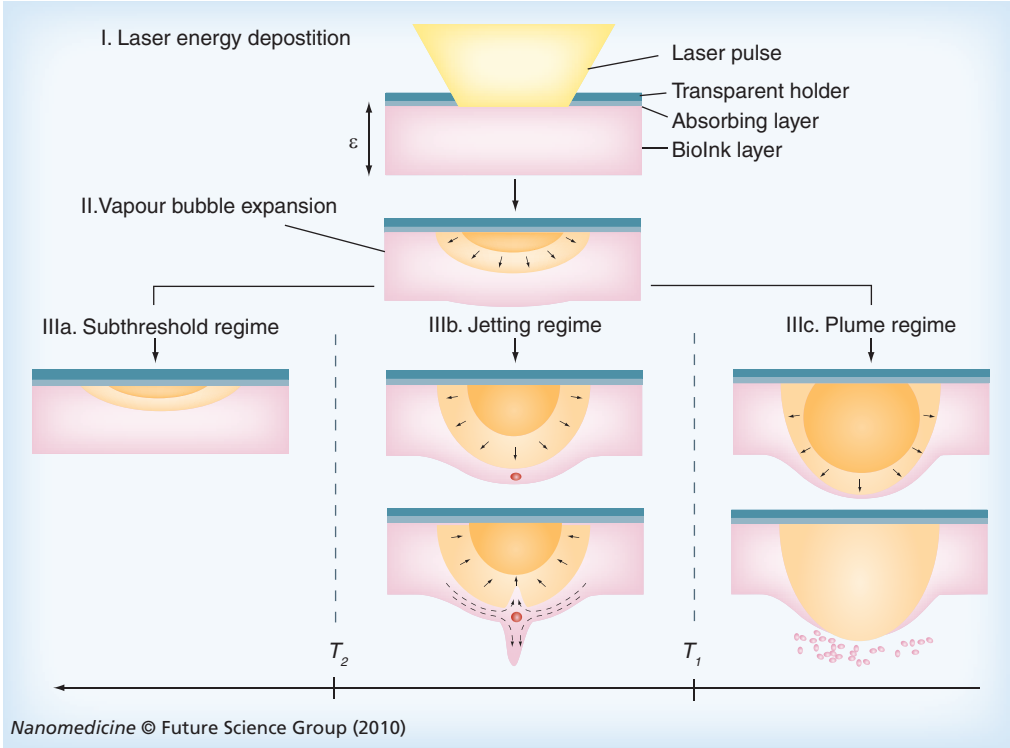


Figure 2. Mechanism for laser-induced droplet ejection. A vapor bubble is generated (see II) by vaporization of the absorbing layer and/or the first molecular layers of the liquid film. At given bioink viscosity and film thickness, jetting (see III.b) is observed for intermediary values of laser fluences ($\Gamma_1 < \Gamma < \Gamma_2$). For a lower fluence ($\Gamma > \Gamma_2$), the bubble collapses far from the free surface without generating a jet (see III.a). For a higher fluence ($\Gamma < \Gamma_1$), the bubble bursts to the surface, generating sub-micrometer droplets (see III.c). Increasing film thickness or bioink viscosity leads to increased threshold Γ values.

Table 1. Key milestones in the development of safe laser-assisted bioprinting procedures.

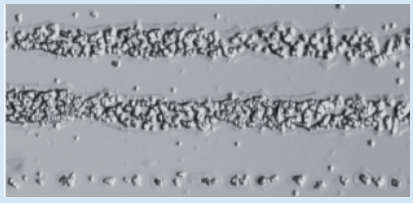
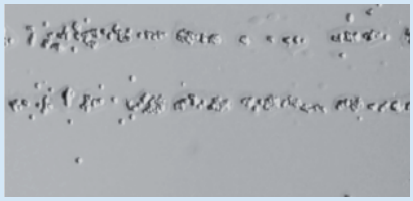
Laser-assisted cell printing method	Key milestones	Ref.
MAPLE-DW, 193 nm	Laser transfer by MAPLE-DW does not damage printed protein epitopes.	[52]
MAPLE-DW, 193 nm, 20 ns, 100 Hz	Transfer technique was found to be compatible with cell transfer as all transferred cells were found to be viable.	[32]
MAPLE-DW, 193 nm, 30 ns	When pluripotent embryonal carcinoma cells (P19) were printed onto a thick layer of Matrigel™ (> 40 µm), more than 90% of the cells survived the transfer process, remained viable and could differentiate into the neural or muscle cell lineage.	[46]
BioLP, 193 nm, 20 ns	A laser absorbing interlayer was placed between the incident laser pulse and biomaterials to be printed. Proof-of-principle experiments of multicolor printing and 3D cell pattern using BioLP (by spreading a 75 µm thick layer of Matrigel between each cell layer).	[30]
BioLP, 248 nm, 2 ns, 100 Hz	Number of cells per spot was shown to be determined by random sampling statistics. Demonstration of minimal expression of heat shock proteins by printed cells.	[53]
AFA-LIFT, 248 nm, 30 ns	Using time-resolved imaging, cell printing was shown to occur on a 1 µs time scale at a constant velocity with an estimated acceleration of 10^7 m/s^2.	[31]
MAPLE-DW, 193 nm, 10 Hz	Co-deposition of hydroxyapatite, MG 63 osteoblast-like cells and extracellular matrix demonstrated the possibility to print complex bioinks.	[54]
MAPLE-DW, 193 nm	Formation of a 3D neural network by transferring B35 neuronal cells to different depths in Matrigel by applying increasing laser fluences (energy per surface; J/m^2).	[55]
MAPLE-DW, 193 nm, 30 ns, 10 Hz, triazene layer	An intermediate layer of absorbing triazene polymer (dynamic release layer) was used to provide a gentler and more efficient printing (i.e., at lower fluences than usual) of B35 neuroblast cells.	[56]
LIFT, 800 nm, 120 fs, 1 kHz	Gap between NIH3T3 fibroblasts containing ribbon and substrate was filled by culture medium to avoid cell stress due to film drying.	[57]
MAPLE-DW, 355 nm, 15 ns	Mammalian embryonic stem cells were printed using a thick polyimide absorbing layer, which differs from a dynamic release layer.	[58]
BioLP, 266 nm, 5 ns, TiO_2 (40 nm)	Glycerol was substituted with methyl cellulose to avoid ink dehydration without toxic effects.	[42]
MAPLE-DW, 93 nm, 300 Hz, triazene DRL	Single-cell printing and groups of cells (approximately 20–40 cells) were obtained when the ribbon was preliminary seeded with a lower cell concentration and near 100% confluence, respectively.	[59]
MAPLE-DW, 193 nm, 2 ns	Some of the MAPLE-DW process-induced damage to yeast cells was reversible and the post-transfer yeast cell recovery was a function of the laser fluence (85–1500 mJ/cm^2)	[44]
Laser-assisted bioprinting, 1064 nm, 5–10 kHz	High-throughout cell printing (up to tens of thousands of droplets per second) was demonstrated using an original workstation. EA.hy926 endothelial cells remained viable after printing using a near-IR nano second laser.	[43]

BioLP: Biological laser printe; DRL: Dynamic release layer; LIFT: Laser-induced forward transfer; MAPLE-DW: Matrix-assisted pulse laser evaporation-direct write.

- If Γ is higher than a threshold value Γ_2 the droplet ejection cannot occur since the bubble expansion is too weak to reach the free surface much; see III.a in Figure 2). This is termed the subthreshold scenario, in which no material can be transferred except if the substrate is in close proximity with the ribbon;
- When Γ is lower than a threshold value Γ_1 the bubble expansion is so violent that it overcomes surface tension resulting in bubble bursting, and hence, liquid splashing onto the substrate; see III.c in Figure 2). This is therefore termed the plume scenario
- If Γ is between Γ_1 and Γ_2: the bubble expands, then collapses and finally a jet is formed; see III.b in Figure 2). Termed the jetting scenario.

Jetting (50 m/s)

By using time-resolved imaging, Duocastella *et al.* recently demonstrate that a long and uniform jet is developed, which advances at a constant velocity (20–150 m/s, depending on experimental conditions) until it reaches the receptor substrate [41]. For the lowest fluences leading to jet formation (e.g., $\Gamma \rightarrow \Gamma_2$), the jet may recoil before reaching the substrate. However, reduction of the gap distance could lead to material deposition onto the receptor substrate. At higher fluences ($\Gamma \rightarrow \Gamma_1$), the jet inertia is high enough to surpass the recoiling force exerted by the surface tension and elasticity of the ink. When a jet has reached a certain length, it becomes unstable and finally breaks due to surface tension effects in the so-called Rayleigh–Plateau instability.

Diaphragm aperture stop (mm)	Laser energy (μJ)
18	
11	16
5	
18	
11	8
5	

Figure 3. Cell printing resolution according to the diaphragm aperture stop (mm) and the laser energy deposit (μJ). The cell concentration of the ink (DMEM, 1% alginate (w/v), 5% glycerol) was 10^8 cell/ml. × 25 magnification.

Deposit: landing

Depending on substrate surface properties, the kinetic energy of the droplet/jet as well as bioink viscosity, droplets collected onto the substrate may exhibit different morphologies, which can be related to splashing and spreading phenomena. This has not been studied in LAB conditions, although it is an important issue in surface science. For moderate initial energies, the surface tension will be able to absorb the initial kinetic energy while for higher energies, the surface tension is not sufficient to stop the outward motion as the drop spreads upon impact, which induces formation of small satellite droplets. In addition, viscosity has been shown to minimize the splashing effect for a given condition of droplet landing onto one substrate [GUILLEMOT *ET AL.* UNPUBLISHED DATA].

Droplets as small as 8 μm have been experimentally produced by reducing the air gap distance and thus working in conditions close to the subthreshold scenario ($\Gamma \rightarrow \Gamma_2$) [33].

Considering safe & high resolution procedures using laser assisted cell printing

Laser-assisted cell printing has been performed in numerous studies over the last five years. Key milestones on the way to developing safe laser-assisted cell printing procedures are reported in TABLE 1. During this period of time, the potential damages caused by the above-mentioned printing steps have been addressed. Besides virus- or microorganism-induced injuries, cell integrity and cell fate might be altered by mechanical, thermal, chemical or/and biochemical stresses. Regarding the LAB process, and considering that sub-threshold and plume scenarios are not compatible with controllable and safe procedures, cell injury may be associated with: bioink composition, interactions of cellular components with light, pressure generated during bubble growth, shear stress within the jet and landing conditions.

Regarding chemical change, biocompatibility of the cell-containing matrices have been addressed with a focus on glycerol content, which is routinely added to avoid dehydration of the bioink caused by the unfavorable surface-to-volume ratio once it is spread onto the ribbon. To limit toxicity induced by glycerol, it has been replaced with methyl cellulose [42]. Also, high-throughput printing may be carried out to reduce evaporation [43]. Indeed, it was recently shown that high speed cell printing (1–100 kHz) is possible and dramatically shortens the printing process [GUILLOTIN B, SOUQUET A, CATROS S *ET AL.*: CELL PRINTING ASSISTED BY LASER DOES NOT PRESENT A COMPROMISE BETWEEN RESOLUTION AND SPEED. UNPUBLISHED DATA].

While calculations have shown that much of the laser energy passes completely through the liquid layer, raising the possibility that the UV laser light could damage the biological structures, it has recently been demonstrated that yeast cells do not suffer from UV irradiation when printed by MAPLE-DW [44]. Nevertheless, this issue remains relevant to *in vivo* printing when body tissues are irradiated for a longer duration [45].

Cell damage due to mechanical stress during laser-assisted cell printing has been observed and is an important issue for further innovations in TE. Mechanical stress can have many origins: increasing hydrodynamic pressure during bubble growth (it was estimated to be a few MPa at a certain distance from bubble front [37]) shear stress during jetting (which depends on jetting speed and thus Γ conditions); and landing conditions (which are governed by the initial jet velocity and the softness and thickness of the substrate). As described above, increasing bioink viscosity or alternatively, reducing laser energy, leads to both reduced bubble expansion velocity and jet front speed. Regarding landing conditions, Ringeisen *et al.* have shown that cell viability is increased to 95% when the substrate is coated by a 40-μm thick Matrigel™ layer [46]. By means of numerical modeling, this result was correlated by Wang *et al.* to a decrease of the first impact-induced stress (von Mises stress) from 3 MPa to 0.86 MPa (for a 50 m/s jet velocity), respectively,

while second impact-induced stress (onto a hard substrate) may also be observed when mattress thickness is less than 40 µm (0.94 MPa for 20-µm coating thickness) [47]. Alternatively or additionally to the landing mattress, a high viability level can also be obtained when a viscous bioink, such as an alginate solution is used [43]. Heat shock protein 60/70 expression has been studied as a potential marker of heat and shear stress sensed by the cells during the printing process [31]. Results show that heat shock protein 60/70 expression is not altered in printed cells compared with control cells. The absence of a detectable effect of LAB on the printed cells has been demonstrated [48]. These data suggest the printed cells may have been exposed to an elevated heat or shear stress for a period of time short enough (few µs) to keep damage below detection thesholds.

Nevertheless, even if safe cell-printing procedures are developed, further studies need to performed to rule out any cell damage, and to determine the effective ratio between the number of printed cells and the number of cells embedded into the ink that are crossed by the laser beam.

Regarding cell printing resolution, LAB sets the benchmark as it is able to print cells one by one, next to each other. Given a 10 µm diameter for a nonadherent cells, maximum cell printing resolution is 1000 cells per cm. Such a resolution has been achieved recently at a high printing speed (5 kHz) (Figure 3) [44]. Although optimization remains possible in terms of higher throughput and higher resolution, such printing resolution and speed are key requirements for cell microarray production or 3D construct fabrication.

Future perspective

In the near future, LAB should be refined thanks to numerical studies that have been recently undertaken [37,38]. Hence, transient values for heat and pressure should be determined and subsequently controlled through manipulation of experimental parameters (e.g., irradiation conditions). Moreover, while jetting conditions have been shown to be subject to a complex threshold mechanism involving energy, viscosity and film thickness, the effects of viscoelastic ink properties on jet formation and recoil should be addressed. Finally, printing conditions without a metallic interlayer should be developed to avoid the potential cytotoxic effect of its residues.

In our opinion, the main issues over the next 5–10 years concerns biological and developmental studies. Developing tools such as LAB would allow us to create and manipulate the *in vitro* cell microenvironment on demand by controlling intensity and shape of cell patterns and morphogen gradients [49,50]. Studies would also deal with generating artificial cell niches by co-depositing a suitable combination of stem cells with extracellular matrix components [51]. In relation to these issues, mechanical and topological cues should be studied using bottom-up approaches for engineering tissues. Combining LAB with other laser-assisted processes, such as machining and polymerization, should be addressed with specific attention on integrating these different processes in the same workstation to guarantee subcellular resolution. Finally, while cell chip fabrication using LAB can be envisaged, other original applications such as medical robotics should be developed in the coming years [46], allowing LAB workstations to leave physics laboratories for biological benches [52–59].

Executive summary

- Regenerative medicine is the process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage or congenital defects.
- Bioprinting consists of computer-aided robotic transfer of living and nonliving biomaterials with the purpose of bioengineering 2D cellular patterns and 3D tissue constructs.
- Laser-assisted bioprinting (LAB) is based on the laser-induced forward-transfer (LIFT) technique in which a pulsed laser is used to induce the transfer of material from a source film spread onto an optically transparent quartz support to a substrate in close proximity to or in contact with the film.
- LAB is a noncontact, nozzle free, high resolution and high speed bioprinting technique. It allows the deposition of volumes smaller than a picoliter, at a micrometer scale resolution and at a meter per second writing speed.
- Jetting conditions are subject a complex threshold mechanism involving laser pulse energy, surface tension and viscoelastic properties of the bioink, as well as the bioink film thickness.
- Printed cell viability and preservation of cellular function are strongly related to hydrodynamics, which govern cell landing conditions onto the substrate.
- Complex multicomponent and 3D printing have already been performed.
- In addition to *in vitro* printing of cells and biomaterials, *in vivo* LAB is also feasible, which offers new opportunities in the field of medical robotics.
- LAB could enable the on-demand creation and manipulation of *in vitro* cell microenvironment by controlling intensity and shape of cell patterns and morphogen gradients.

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Special Report

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Regenerative Medicine

Operating RegenMed: development of better in-theater strategies for handling tissue-engineered organs and tissues

Tissue engineering *ex vivo* and direct cellular application with bioscaffolds *in vivo* has allowed surgeons to restore and establish function throughout the human body. The evidence for regenerative surgery is growing, and consequently there is a need for the development of more advanced regenerative surgery facilities. Regenerative medicine in the surgical field is changing rapidly and this must be reflected in the design of any future operating suite. The theater environment needs to be highly adaptable to account for future significant advances within the field. Development of purpose built, combined operating suites and tissue-engineering laboratories will provide the facility for modern surgeons to treat patients with organ deficits, using bespoke, regenerated constructs without the need for immunosuppression.

Keywords: biotechnology • operating room • operating theater • reconstruction • regeneration • regenerative medicine • scaffold • stem cells • surgery • tissue engineering

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Background

Significant advances have been made in regenerative medicine ('RegenMed') over the past decade and the field is advancing at a rapid pace. RegenMed now permeates through every surgical specialty [1,2]. The combination of tissue engineering *ex vivo* and direct cellular application with bioscaffolds *in vivo* has allowed modern surgeons to restore and establish function throughout the human body from replacing airways, the GI tract, hepatobiliary system, myocardium, kidneys, urinary tract to the skin, bone and connective tissues [3–8].

Although a proportion of the literature surrounding regenerative procedures is based on preclinical laboratory work, the clinical evidence for regenerative surgery is undeniably growing, and coupled with this is the need for the development of more advanced regenerative surgery facilities [9]. Achieving these advances in surgical science has required highly qualified, large multidisciplinary teams and advanced tissue-engineering facilities.

Alongside the scientific trials have been the ongoing debates regarding the ethical and practical issues surrounding the field of stem cell and animal research [10]. As a consequence, this expanding area of surgery has been limited to quaternary centers with substantial funding and international expertise. The future of regenerative surgery will be the development of specific operating theaters that have the capacity for on-site tissue engineering, with stem cell acquisition, scaffold generation and direct graft implantation, all within reach of the multidisciplinary team performing the operation.

Methods

We conducted a PubMed and ClinicalTrials.gov review of the literature surrounding RegenMed, tissue engineering and their role in the surgical field using variants of the search terms 'regenerative medicine', 'regenerative surgery', 'stem cells', 'tissue engineering', 'tissue transplantation' and 'biotechnology' from database inception to 10 May 2014. We used Boolean operators to refine our search and included all articles regardless of date and article type in the initial search. Articles were analyzed and selected based on

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the relevance to the topic of interest and were excluded if they were not in English or were duplicate in material covered. Review articles were preferred to original papers in topics where there was more substantial literature, whereas primary research articles were preferred in more innovative areas. The references of relevant articles were analyzed for further material. Information was included from basic science research, completed early-phase clinical trials and on-going clinical trials (Figure 1).

Principles of RegenMed

RegenMed replaces or regenerates human cells, tissues and organs, to restore or establish normal function [11]. The regeneration of tissue can take place either *in vivo* or *in vitro*, and may utilize stem cells, natural or synthetic bioscaffolds and bioactive molecules to induce cell differentiation, proliferation and genetic manipulation. [2] In reality, most modern techniques involve a combination of techniques depending on the end goal of the regenerative procedure [12].

Cells

Autologous cells are used most often for regenerative purposes (because they are not rejected), although cells can be sourced from allogenic or xenogenic donors. Ideally, they must be nonimmunogenic, highly proliferative, easy to harvest and possess the ability to differentiate into a variety of cell types with specialized functions [13]. Acquisition of stem cells for clinical regenerative purposes can be from several sources and these may be totipotent, pluripotent or tissue-specific, including mesenchymal stem cells (MSCs) from bone marrow/adipose tissue/umbilical cord/placenta,

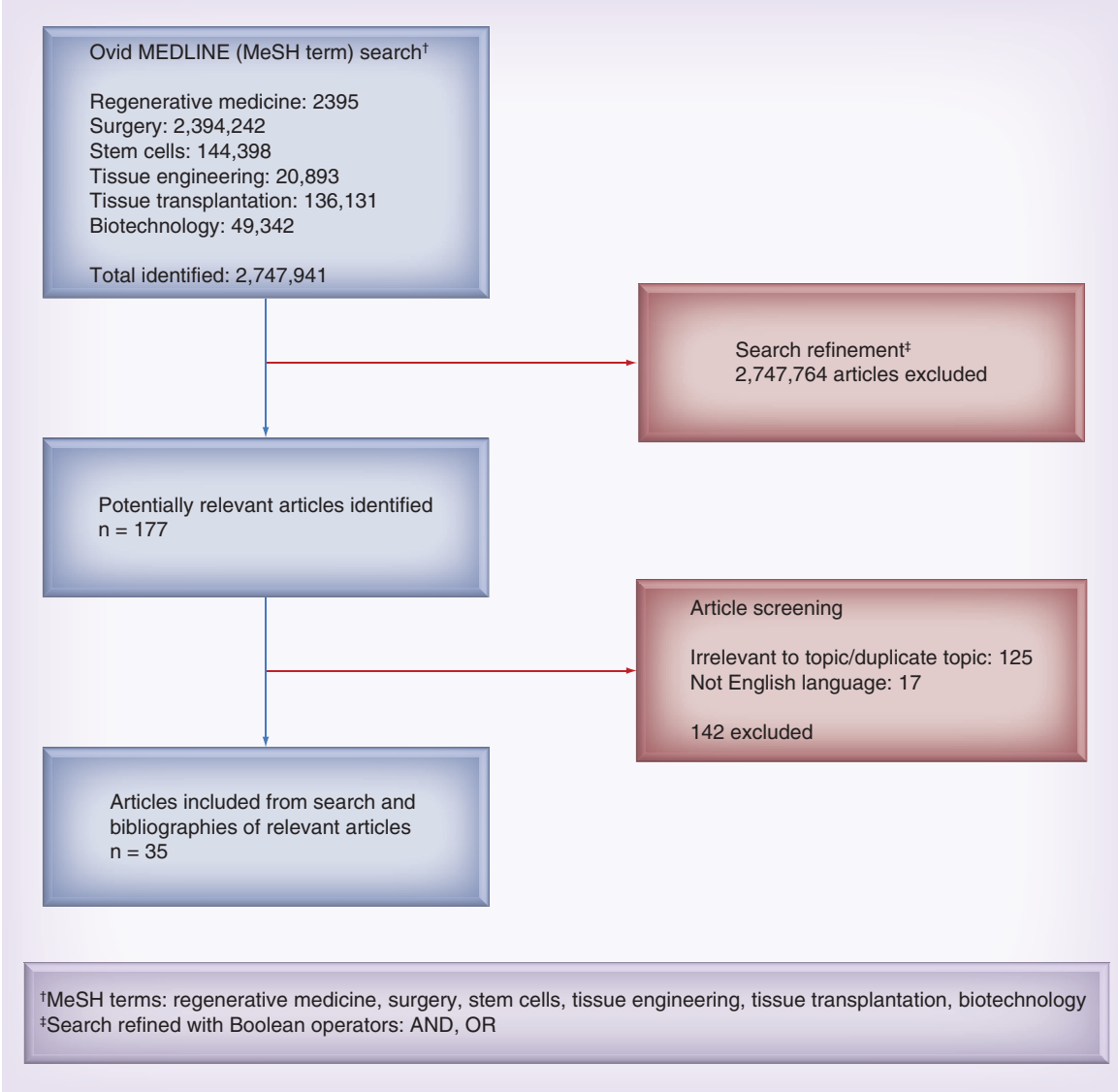

Figure 1. PRISMA flow chart depicting the search strategy that was employed in the generation of this review.

satellite cells, muscle stem cells, embryonic stem cells, induced pluripotent stem cells or amniotic fluid stem cells [14–16]. Many of these have already satisfied preclinical criteria and are being tested in clinical trials, or are in late preclinical phase. Hare and colleagues used MSCs to promote ventricular remodeling in patients with ischemic cardiomyopathy [17]. Cossu and colleagues are currently conducting a clinical trial of mesangioblast implantation for treatment of Duchenne's muscular dystrophy, while we and our colleagues are commencing a UK-based trial of tissue engineered laryngeal replacements for patients postlaryngectomy funded by the UK MRC (RegenVOX) [18,19].

Amniotic fluid stem cells have particular relevance in the prenatal generation of tissue for children who have been with congenital abnormalities *in utero*, allowing immediate graft implantation following birth [6]. The choice of specific stem cell to use depends on the tissue that is going to be replaced by the procedure; for example, whereas one cell type may be better for developing tissue for reconstruction of the trachea, a different cell is likely to be needed for constructing heart valves.

There is growing evidence that tissue-engineered cardiac muscle can be used to restore function following myocardial infarct in rats. In one study, tissue-engineered heart tissue, harvested from neonatal rat heart cells, prevented further dilation, induced systolic wall thickening of infarcted myocardial segments and improved fractional area shortening of infarcted hearts compared with controls [20].

Scaffolds

The prime requirements for any scaffold in tissue engineering are biocompatibility, nonimmunogenicity, the capacity to sustain and/or promote the growth of the relevant cells/tissue, and provision of a template for tissue growth in three dimensions. Many different materials (natural and synthetic, biodegradable and permanent) have been investigated. Synthetic materials offer strength, processability, degradation, microstructure and permeability, but can predispose to inflammation and foreign body reactions. The most commonly used synthetic scaffolds are polyglycolic acid structures, sometimes in combination with poly-4-hydroxybutyrate [21]. Natural scaffolds can be formed from the *in vivo* extracellular matrix components and, thus, have intrinsic interactive properties such as cell adhesiveness and enhanced biocompatibility [12,13]. However, natural materials are prone to attack from the host immune system, leading to inflammation and fibrosis and distortion of the form and function of the graft. Some of these limitations can be addressed using synthetic scaffolds. However, there are other challenges associated with synthetic scaffolds, such as designing optimal microstructures to support cell growth and achieving a balance between polymeric degradation and tissue formation [22].

Various different decellularization methods have been employed to date, including both physical and chemical methods [13]. The detergent–enzymatic decellularization method has made it possible to create a biocompatible acellular matrix, whereby cells can offer improved survivability, preserved structural integrity, biomechanical advantages, extracellular matrix stability, and better cell adhesion and differentiation, and has been shown to modulate both humoral and cell-mediated immune responses, thereby minimizing immune recognition and classical transplant rejection [22–26]. Thus, patients have received tissue-engineered airways from decellularized allogenic scaffolds and, to date, have not developed anti-HLA antibodies to date in the absence of immunosuppression [27–29]. However, the process of decellularization is not applicable to all tissues, most notably for the development of aortic valves, where biopolymers are more suitable.

Tissue generation

The first stage in the process of *in vitro* tissue generation to replace lost or inadequate tissue involves cell harvesting from a biological source containing stem cells, for example bone marrow. The stem cells should then be isolated from the sample and expanded in cell culture. Following this, the *in vitro* expansion of the stem cell population takes place in a bioreactor to stimulate proliferation and differentiation of specific cell populations, as well as adhesion to the scaffold. Most of the early work in tissue engineering used standard static cell-culture conditions for the *in vitro* fabrication of tissue before implantation. Since then, the introduction of bioreactors has enabled the *in vitro* culture of greater volumes of cells by producing a dynamic microenvironment culture system. Bioreactors, by flow and mixing, can ultimately facilitate cell integration and growth inside the scaffold or matrix by amplifying the mass transfer of nutrients, gases, metabolites, and regulatory molecules and also by providing mechanical stimulation that can induce specific cellular adaption [12–13,30]. Finally, once the autologous graft has been generated, it can be implanted into the patient to restore lost tissue or establish functionality.

Routine cardiac valve replacement currently relies on the use of mechanical valves with formal, lifelong anticoagulation or porcine tissue valves with limited longevity. Tissue-engineered valves have been generated using both synthetic and biological scaffolds and, by remodeling and regeneration, are able to overcome these limitations [31].

Macchiarini *et al.* successfully applied an *in vitro* tissue-engineering process using a donor trachea, which was decellularized and then readily colonized by the recipient's epithelial cells and chondrogenic MSCs [28]. Importantly, the nature of the patient's disease in this case made *in vitro* (thus delayed) preparation of the tissue-engineered organ possible with subsequent implantation.

For *in vivo* tissue generation, the initial cell harvesting, isolation and preparation are the same. The cells are then directly infiltrated onto a scaffold and *in vivo* cell seeding and adhesion takes place under the influence of cytokines and biochemical to promote differentiation [12]. In comparison to the work by Macchiarini and colleagues, Elliot and colleagues describe a case where the need for intervention was more immediate and, thus, an *in vivo* method was adopted. In this situation, a child required emergency implantation of a neotrachea. By using a decellularized cadaveric trachea seeded with autologous MSCs and exposed to topical biochemical inducers of differentiation, the airway was successfully reconstructed with total functionality at 2-years postoperation [27].

RegenMed operating theater: design & practicalities

Our concept of the future design and practicalities of creating an optimized operating suite for RegenMed is based on our prior experience of transplanting tissue-engineered constructs into experimental animals and children with unmet clinical needs [27]. Currently, Tengion Inc. (NC, USA) has been a pioneer in the regenerative surgery with engineered constructs. Their system involves the harvesting of stem cells in the hospital setting, with the cell-culture and tissue-engineering steps taking place off-site [32]. Although this system has been successful up to a point, the next stage will encompass the entire regenerative and reconstructive process being performed at one site. Alternatively, adipose-derived and bone marrow-derived MSCs can be procured intraoperatively (e.g., Celution® from Cytori Inc. [CA, USA], or MarrowXpress™ from Celling Bioscience [TX, USA]) and used as suspensions of mononuclear cells or in combination with scaffold materials, such as demineralized bone.

The future RegenMed Operating Suite will be equipped to harvest autologous stem cells from patients immediately prior to surgery. An example method for this would be to harvest a sample of bone marrow aspirate, or adipose tissue, from the patient. The suite should house a centrifuge, cell isolation apparatus and cell culture facilities that would allow the team to immediately isolate MSCs from the sample. Cells should be stored in optimal conditions (37°C, 5% CO_2) in an incubator, housed within the operating theater for *in vivo* tissue regeneration, or in the adjacent laboratory for seeding onto scaffolds for *in vitro* tissue regeneration. Sanyo™ (Osaka, Japan) have developed an integrated cell processing workstation that enables cell culture and manipulation in an efficient, aseptic and cost-effective way without the need for a dedicated clean room [33]. This kind of workstation could be integrated into the design of the RegenMed theater, either within the suite or immediately adjacent, allowing the team to harvest, culture and apply autologous stem cells to the bespoke scaffold within the same aseptic procedure. All facilities would be required to be compliant with EU GMP standards and, at present, to be licensed by the Medicines and Healthcare Products Regulatory Agency in the UK as a pharmaceutical manufacturing site. This is a major undertaking and will require substantial resources, which will exceed the costs of running a purpose-built RegenMed theater. This is a rapidly changing field and regulators around the world are already considering how operating theaters can become satellites of licensed GMP manufacturing laboratories (Figure 2).

It is likely that *in vivo* tissue engineering will be better suited to this type of set-up as it would prevent the need for multiple procedures, both respect to harvesting stem cells and transplanting the tissue-engineered construct. Alternatively using an *in vitro* approach, a dedicated RegenMed laboratory adjacent to the operating theater containing a bioreactor would be optimal so as to facilitate timely generation of the organ tissue and it is safe transfer to the patient at the time of surgery. The RegenMed theater itself would need to have facilities in place to store a variety of scaffold materials, in order to accommodate the vast variability in application and suitability of both natural and synthetic products. Ideally, the suite should also house apparatus to decellularize cellular matrices, such that bespoke scaffolds can be generated *ad hoc*.

Several bioactive agents are currently available for the generation of improved regenerated grafts. Granulocyte-colony stimulating factor has historically been used to stimulate proliferation of progenitor cells before bone marrow cell harvest and transplant, but has more recently been found to augment MSC recruitment into a bioscaffold [34]. Human recombinant erythropoietin may improve survival of cells in tissue that is oxygen depleted due to immature angiogenesis [35]. TGF-β has been shown to potentiate differentiation of MSCs into chondrocytes, but also promotes myofibroblast-mediated scar formation [27,36]. The RegenMed theater would have the facility to utilize a range of these biomolecules, ready to be applied to the tissue-engineered construct, thereby allowing a variety of tissues to be generated, depending on the requirements of the individual patient.

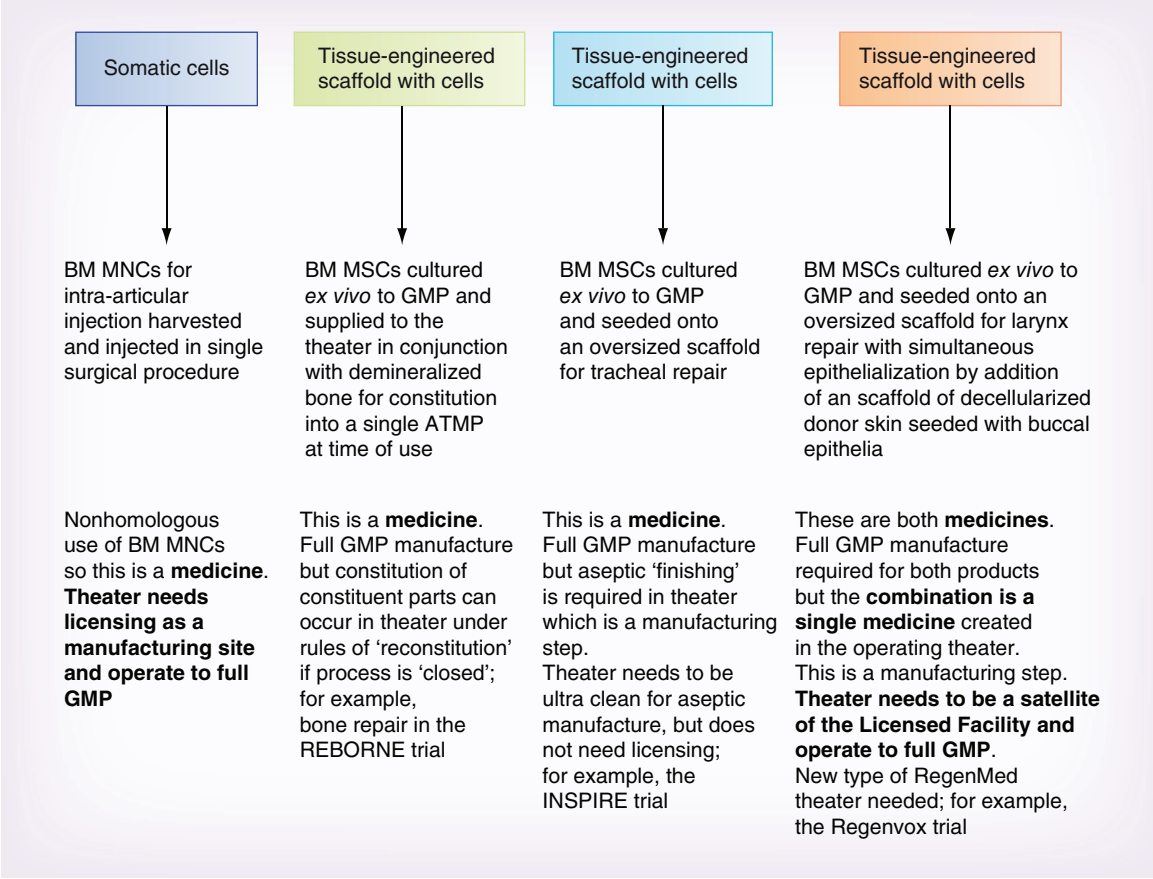

Figure 2. GMP compliance of various tissue-engineered therapies in clinical practice.
ATMP: Advanced therapy medicinal product; BM: Bone marrow; MNC: Mononuclear cell; MSC: Mesenchymal stem cell; RegenMed: Regenerative medicine.

Several other important aspects of the RegenMed theater need to be mentioned. First, is the ultraclean nature required for the aseptic processes – class 100 is required compared with conventional class 10,000 operating theaters (i.e., 100-times cleaner). Second, imaging equipment, with respect to monitoring of the graft during implantation requires: cell and tissue labeling with dyes and nanomarkers for super high-resolution CT and MRI, or bioluminescence akin to Luminex technology used in animal studies. Progress is required in this area. Third, the capacity for a multidisciplinary team working in a segmented theater design to maintain an aseptic cordon around the patient is required that would still allow highly specialized radiology and near-patient product finishing by GMP scientists within the theater complex. Open theaters with invisible air showers between multidisciplinary team groups could be one solution to overcome this.

Finally, the role of robotics and 3D imaging in the operating theater is an ever-growing area; its application is broad, but is predominantly utilized in urological and gynecological surgery. Although its role is distinct from that of RegenMed, they are both pushing the frontiers of modern surgery and for this reason, it would be prudent of the RegenMed operating theater to have adaptations that would allow surgical robotics to be easily integrated into its design, thereby leading to the creation of hybrid operating rooms capable of sustaining multiple technological advances in the forthcoming years [37–39].

Conclusion & future perspective

The field of RegenMed, as applied to surgical disease, is a rapidly changing field and this would need to be reflected in the design of any future operating suite with RegenMed in mind. The theater needs to be highly adaptable to account for significant advances that we can expect within the field over the next few years. There are still many unanswered questions, which will necessitate further laboratory-based research in the shorter term, with formal clinical trials in the longer term [40]. However, undeniably surgical intervention, in combination with the science of tissue engineering and RegenMed, can provide a definitive surgical cure for many conditions for which there is no current treatment strategy.

Current operating theaters are poorly equipped to deal with the scientific advances in tissue engineering. Development of purpose built, combined operating suites and tissue-engineering laboratories will provide the facility for the modern surgeon to treat patients with tissue or organ deficits, using bespoke, regenerated constructs without the need for immunosuppression.

Financial & competing interests disclosure

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Executive summary

Premise

- Significant advances have been made in regenerative medicine (RegenMed) over the past decade and the field is advancing at a rapid pace.
- The combination of tissue engineering *ex vivo* and direct cellular application with bioscaffolds *in vivo* has allowed modern surgeons to restore and establish function throughout the human body.
- The evidence for regenerative surgery is undeniably growing, and coupled with this is the need for the development of more advanced regenerative surgery facilities.

RegenMed operating theater

- Our review of the literature surrounding the use of regenerative technologies in the surgical field, establishes the current advancements, the future direction of this field and how the modern operating theater can incorporate technology to facilitate the implementation and development of regenerative surgery.
- The field of RegenMed, as applied to surgical disease, is a rapidly changing field and this would need to be reflected in the design of any future operating suite with RegenMed in mind.
- The theater needs to be highly adaptable to account for significant advances that we can expect within the field over the next few years; current operating theaters are poorly equipped to deal with the scientific advances in tissue engineering.

Conclusion

- Development of purpose built, combined operating suites and tissue-engineering laboratories will provide the facility for the modern surgeon to treat patients with tissue or organ deficits, using bespoke, regenerated constructs without the need for immunosuppression.

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Advanced nanobiomaterial strategies for the development of organized tissue engineering constructs

Nanobiomaterials, a field at the interface of biomaterials and nanotechnologies, when applied to tissue engineering applications, are usually perceived to resemble the cell microenvironment components or as a material strategy to instruct cells and alter cell behaviors. Therefore, they provide a clear understanding of the relationship between nanotechnologies and resulting cellular responses. This review will cover recent advances in nanobiomaterial research for applications in tissue engineering. In particular, recent developments in nanofibrous scaffolds, nanobiomaterial composites, hydrogel systems, laser-fabricated nanostructures and cell-based bioprinting methods to produce scaffolds with nanofeatures for tissue engineering are discussed. As in native niches of cells, where nanofeatures are constantly interacting and influencing cellular behavior, new generations of scaffolds will need to have these features to enable more desirable engineered tissues. Moving forward, tissue engineering will also have to address the issues of complexity and organization in tissues and organs.

KEYWORDS: bioprinting ■ hydrogel ■ laser biofabrication ■ nanobiomaterial ■ nanofiber

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Tissue engineering and regenerative medicine are promising new therapies to meet the global challenge of tissue/organ shortage [1]. However, the philosophy of tissue engineering and regenerative medicine varies considerably with the expertise of individual investigators, some use a biodegradable scaffold while others do not [2]. Currently, the speed of vascularization for implanted engineered tissues is generally low, and viable tissues that can be created, either *in vitro* or *in vivo*, are limited to structurally thin and relatively simple tissues such as skin, cartilage and bladder. Moreover, it is not unusual for the mechanical properties of engineered tissues to be inferior to their native counterparts. It is generally believed that the overall characteristics of an engineered tissue must result from its unique composition and organization of microstructures, such as the organization of cells and extracellular matrices, and that the problem of vascularization and inferiority are likely to be due to the microscale materials and structures within the tissue. Therefore, engineering extracellular matrices and promoting rapid formation of the cellular microenvironment is essential for advancing current tissue engineering and regenerative medicine. The building blocks of extracellular matrices are primarily nano- and micro-scale biomaterials that are dynamically synthesized, organized, remodeled and eliminated by cells. Their temporary presence in tissues usually allows direct physical contact with cell surface receptors, initiating an intracellular cascade of chemical reactions that eventually lead to various phenotypic behaviors such as adhesion, spreading, migration, DNA and protein synthesis, proliferation, senescence, apoptosis, orientation, and alignment. These nano- and micro-scale biomaterials mediate the microenvironment and cellular responses. Correct utilization of these materials can potentially unclock the code of cellular language and instruct cells to release their veiled potential for tissue repair and organ reconstruction.

Nanobiomaterials, at the interface of biomaterials and nanotechnology, refer to a special class of biomaterials with constituent or surface sizes less than 100 nm [3]. Their fine structure allows direct mechanical interactions with cell surface receptors and cellular components, and hence manipulation of cells to serve intended diagnostic or therapeutic purposes. Particularly when applied to tissue engineering and regenerative medicine, nanobiomaterials are usually perceived as microenvironment-like substances in which rich extracellular matrices and various cell types, including stem cells, reside. Nanobiomaterials, when applied to tissue engineering, are usually perceived as having a close resemblance to the microenvironment where cells reside. Through their interaction with cells, nanomaterials act as a means of providing instructive signals to the internal architecture of a cell.

There have been a number of attempts to engineer 3D tissues, but little progress has been made

Future Medicine part of fsg

on engineering 3D organized tissues (FIGURE 1). It is generally believed that the third dimension of an engineered tissue can not exist alone for a long period of time if there is no order being created at the nano- or micro-scale within the tissue. Therefore, it is of paramount importance to acquire further knowledge of nanobiomaterials in order to bridge the gap between biomaterials and nanotechnology, and to reveal their full potential for tissue engineering and regenerative medicine. This review discusses some recent progress on nanobiomaterial strategies in the field of tissue engineering and regenerative medicine, focusing on those with the potential for developing 3D organized tissue engineering constructs.

3D nanofibrous scaffolds & nanocomposites

Nanomaterials are widely utilized in tissue engineering and regenerative medicine because they are able to mimic compositions [4], topographies [5] and architectures [6] of human tissues, and may offer enhanced or new properties to artificial constructs [7]. They have been fabricated into various basic structural units, such as nanoparticles, nanocrystals, nanofibers and nanofilms, to fulfil the specific requirements of biological substitutes that repair or replace malfunctioning tissues [8]. Nanofibrous scaffolds, especially 3D scaffolds, have attracted considerable attention in tissue regeneration in recent years, mainly due to their structural similarity to native extracellular matrix, applicability to a wide range of materials, and readily tunable fiber size and spatial arrangement [9]. To date, nanofibrous scaffolds have been applied in research and regeneration of various tissues (e.g., skin [10,11], vascular [12], bone [13,14], cartilage [15], bladder [16], neural [17,18] and cardiac tissues [19]) *in vitro* and, more significantly, *in vivo*. 2D mats and 3D cotton-like balls are the two typical configurations used for nanofibrous scaffolds. In a recent study, Hsiao *et al.* fabricated an aligned 2D conductive nanofibrous mesh with poly(lactic-*co*-glycolic acid) and polyaniline to induce elongated and aligned rat cardiomyocyte clusters with synchronous cell beating [20]. However, 2D mats are less favorable unless the orientation or functionality of nanofibers is of great importance [21,22]. 2D mats, especially those that are electrospun, usually have a flat topography and tightly packed fibers that restrict cell infiltration to the superficial layers of the scaffolds and cellular integration with host tissue after implantation. Cell sheet technology transforms 2D nanofibrous mats into 3D functional tissues by stacking individual 2D confluent cell sheets recovered from thermoresponsive culture substrates [23,24]. In this manner, it extends the

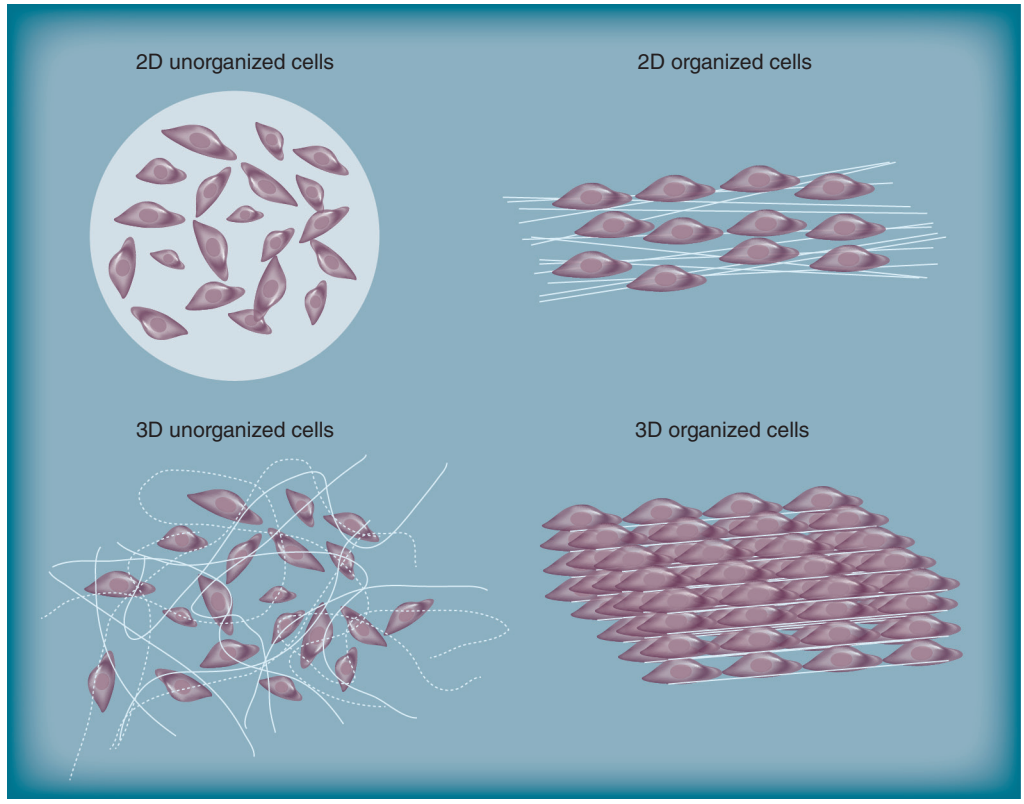

Figure 1. Organization of cells in engineered tissues.

application of 2D mats into producing implantable 3D tissues [25], but its potential will not be fully exploited until good nutrient transportation is achieved in thick cell stacks. Therefore, 3D nanofibrous scaffolds with proper pore size and interconnectivity are highly desirable to enable satisfactory cell infiltration and nutrient diffusion. Electrospinning is the most commonly used technique to fabricate 3D nanofibrous scaffolds with uniform morphology and stability; some are coupled with micrometer-sized framework [26,27] and some are directly electrospun [28,29]. Blakeney *et al.* devised a novel electrospinning collector that is an array of metal probes radially arranged in a spherical foam dish to harvest cotton ball-like poly(ε-caprolactone) scaffolds between the metal probes in mid-air (FIGURE 2). The resulting poly(ε-caprolactone) scaffolds were highly porous and cell infiltration was significantly improved [28]. In another study, Bonino *et al.* reported that 3D alginate nanofiber mats can be electrospun via charge repulsions from negatively charged ions dissociated by the carboxylic acid groups of alginate [29]. In addition to the methods mentioned above, a number of post-electrospinning techniques, such as polymer/salt leaching and laser/UV irradiation, are harnessed to improve the porosity of as-spun scaffolds [30]. Moreover, surface functionalization of electrospun fibers and drug encapsulation with nanofibers can further tailor the nanofibrous scaffolds to improve their performance, for example, by facilitating cell adhesion, spreading and growth, and controlled release of drugs [31].

In order to enhance certain properties or create new functionalities, multicomponent materials may be used to fabricate scaffolds [32]. On top of functional polymers such as electrically conductive polymers, hydroxyapatite, metal nanoparticles and carbon nanomaterials (e.g., fullerenes, carbon nanotubes and graphene) are often incorporated into polymeric matrix to fabricate nanocomposites for applications of tissue engineering and regenerative medicine. Hydroxyapatite, a biocompatible ceramic material mainly used in bone tissue engineering, is capable of resembling bone minerals in morphology and composition [33] and, thus, is extensively employed as part of nanocomposites in bone tissue engineering [34,35]. Electrically conductive materials are usually doped into a polymeric matrix to make conductive fibers/films for stimulating neurons and, hence, neural tissue repair [21]. In a recent study, aligned carbon nanotubes, rolled-up graphene sheets with excellent mechanical and electrical properties, were coated with para-toluene sulfonic acid-doped polypyrrole to form a novel nanostructured conductive platform, in which carbon nanotubes provided the topography and para-toluene sulfonic acid-doped polypyrrole provided the biocompatibility. It has been reported that the rate of differentiation and cell division of primary myoblasts cultivated on the conductive nanocomposite films can be controlled by electrical stimulation [36]. However, the potential toxicity of carbon nanomaterials has always been emphasized [37,38] and, although many *in vitro* experiments have demonstrated that they are nontoxic, the scientific community has to be fully convinced before any significant clinical applications can be realized [39,40]. Metals possess unique physical, chemical and biological properties when downsized to the nanometer scale compared with their macroscopic states. For instance, silver nanoparticles have been used for antibacterial applications. Agarwal *et al.* precisely controlled the loading of silver nanoparticles in thin polymeric films to allow antimicrobial activity without inducing cytotoxicity in mammalian cells [41]. Similar to carbon nanomaterials, the potential risk of metal nanoparticles should be fully understood and controlled before they are utilized in clinical applications.

Hydrogels

Hydrogels consist of a network of crosslinked polymer chains with the ability to absorb large amounts of water without disintegrating. This makes hydrogels unique and attractive as nanobiomaterials for tissue engineering and drug delivery applications. Hydrogels, including thermoresponsive and pH-sensitive gels, have

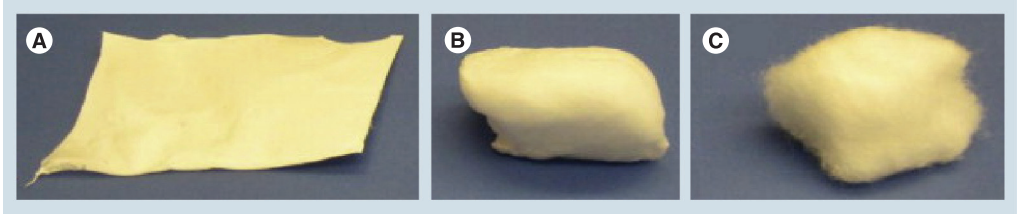

Figure 2. Electrospun nanofibrous scaffolds. (A) 2D nanofibrous scaffolds and **(B & C)** 3D nanofibrous scaffolds.

been researched extensively. Some of the more recent advancement in hydrogels for engineering 3D organized tissues will be reviewed. TABLE 1 shows desired properties of a hydrogel scaffold for 3D organized tissues and currently available strategies to achieve them.

In recent years, bioresponsive hydrogels have progressed substantially, bringing the engineering of a 3D organized tissue a step closer. One of these developments is spatially bioactive hydrogen. In work by Zhu *et al.*, a biomimetic hydrogel scaffold with controlled spatial organization of nanobiomaterials, such as cell-adhesive ligands, was developed [42]. Cyclic Arg–Gly–Asp peptides were first attached in the middle of poly(ethylene glycol) diacrylate (PEGDA) chains and hydrogel formation was initiated via photopolymerization. The authors showed that cyclic Arg–Gly–Asp–PEGDA hydrogels could facilitate endothelial cell adhesion and spreading, and exhibited significantly higher endothelial cell proliferation compared with linear Arg–Gly–Asp-modified hydrogels at low peptide incorporations. Incorporation of cell-adhesive ligands and controlling ligand density and spatial organization is an initial but critical step for hydrogels to be three-dimensionally responsive to cellular adhesions. Stimulation of microenvironmental factors, such as electrical signals, has also been shown to be important because some tissues, such as muscles, require electrical stimuli to function. Mawad *et al.* developed a single component, conducting hydrogel by covalently crosslinking a poly(3-thiopheneacetic acid) hydrogel with 1.1′-carbonyldiimidazole [43]. In addition to swelling ratios up to 850%, the hydrogels were shown to be electroactive and conductive at physiological pH. In terms of cellular responses, fibroblast and myoblast cells were able to adhere and proliferate well on the hydrogel substrate.

One of the common problems with hydrogels is their poor mechanical properties. To enhance the mechanical properties of hydrogels, incorporating nanobiomaterials into hydrogels could be a possible solution. As shown by Kai *et al.*, incorporation of poly(ε-caprolactone) nanofibers into gelatin hydrogel resulted in the increase of the Young's modulus of the composite hydrogels from 3.29 to 20.3 kPa. [44]. Wu *et al.* studied the photocrosslinking of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) triblock copolymer diacrylates (Pluronic® F127 diacrylate; BASF, Ludwigshafen, Germany) in the presence of the silicate nanoparticle Laponite® (Rockwood Additives, TX, USA) and the resulting hydrogels had high elongations and improved toughness [45]. Chang *et al.* developed PEGDA/Laponite nanocomposite hydrogels, and the incorporation of Laponite nanoparticles significantly enhanced both the compressive and tensile properties of PEGDA hydrogels [46]. The authors also demonstrated that their nanocomposite hydrogels were able to support 3D cell culture. In addition to mechanical advantages, incorporation of nanobiomaterials also offers bioactive advantages. Azami *et al.* prepared a gelatin–amorphous calcium phosphate nanocomposite scaffold that has a three-dimensionally interconnected porous microstructure. After incubation in simulated body fluid solution at 37°C for 5 days, the mineral phase of the scaffold was transformed into nanocrystalline hydroxyapatite [47]. Sowmyaa *et al.* reported a chitin hydrogel scaffold lyophilized with bioactive glass ceramic nanoparticles, which was found to have enhanced porosity, swelling, bioactivity and degradation [48]. Moreover, the composite scaffolds were nontoxic to human osteoblasts and suitable for periodontal bone defects. Sudheesh Kumar *et al.* developed chitin/nanosilver composite scaffolds that were effective against *Escherichia coli* and *Staphylococcus aureus* [49].

Spatially controlled release of growth factors is a desired property of a tissue engineering scaffold, and this may be conveniently realized after the invention of nanogels. Nanogels are a special type of hydrogel in which hydrogel nanoparticles or nanogels (<100 nm) are either chemically or physically crosslinked by polymer chains to form a 3D network [50]. Owing to their nanometer size, nanogels are more effective at stably trapping bioactive compounds inside their network and respond more rapidly to microenvironmental factors such as temperature and pH. Therefore, nanogels are important for spatially controlled release of growth factors within a scaffold. FIGURE 3 shows a schematic of the preparation of a cholesterol-bearing pullulan nanogel-crosslinking hydrogel to deliver BMP-2 [51]. Hayashi *et al.* examined the efficiency of

Table 1. Nanobiomaterial strategies for enhancing the properties of hydrogels.

Desired properties of hydrogel scaffold	Nanobiomaterial strategies
Bioresponsiveness	Nanoscale ligands
Mechanical strength	Nanocomposites
Controlled release of growth factors	Nanogels
Properties of native proteins	Self-assembled peptides

nanogels to deliver BMP-2 *in vivo* for bone defect repair. Despite a single implantation with low amounts of BMP, vigorous osteoblastic activation and new bone formation were evident [51]. Kamolratanakul *et al.* went further by delivering a combination of a selective EP4 receptor agonist and a low dose of BMP-2 in a nanogel-based disc scaffold, and observed efficient activation of bone cells and effective regeneration of bone tissues [52]. In another study, Bencherif *et al.* hybridized nanogels to hyaluronic acid by mixing them under physiological conditions (pH = 7.4; 37°C), and created a nanostructured hyaluronic acid hydrogel scaffold with a porous 3D uniform distribution of nanogels [53].

In addition to the aforementioned properties, there is considerable interest in developing self-assembled peptide nanostructure to

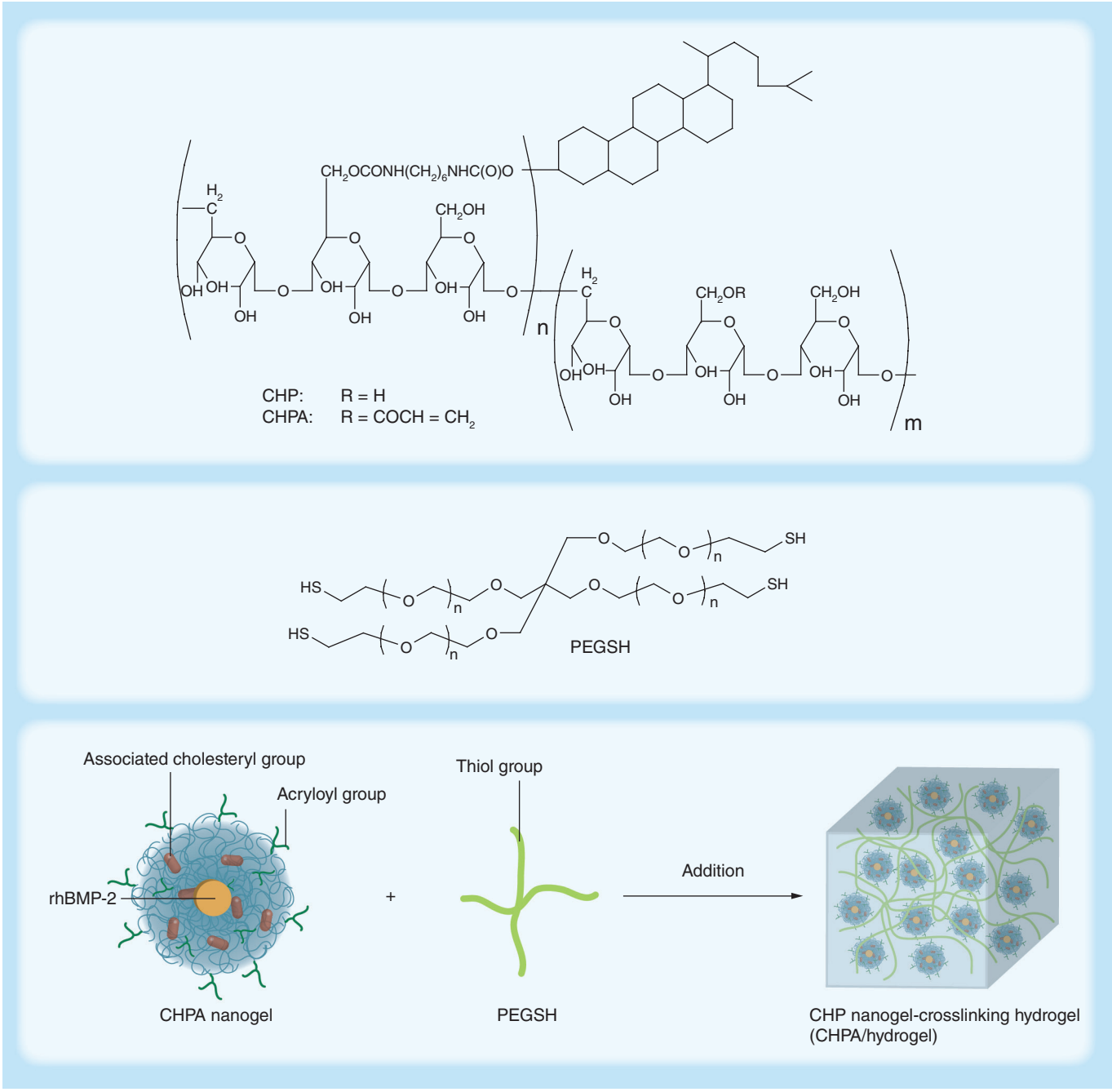

Figure 3. Acryloyl group-modified cholesterol-bearing pullulan nanogel-crosslinking hydrogel containing BMP-2 growth factor.
CHP: Cholesterol-bearing pullulan; CHPA: Acryloyl group-modified CHP; PEGSH: Thiol group-modified poly(ethylene glycol); rhBMP-2: Recominant human BMP-2.
Reproduced with permission from [51].

mimic the creation process of native proteins. O'Leary *et al.* designed a peptide sequence (Pro–Lys–Gly)$_4$(Pro–Hyp–Gly)$_4$(Asp–Hyp–Gly)$_4$ that can form a stable triple helix and replicates the self-assembly of collagen through all steps. The resulting nanofibres can form a hydrogel that is degraded by collagenase at a similar rate to that of natural collagen [54]. The ability to design and synthesize peptides with characteristics that are similar to their native counterparts can offer significant advantages in the control and manipulation of scaffold properties.

Laser-fabricated 3D nanostructures

Laser technology is able to generate fine features, such as ridges, grooves and standing rods, among others, on a 2D surface, and it has shown remarkable influence on various cell behaviors, including cell attachment, orientation, proliferation and differentiation [55–58]. Most, if not all, of these studies are conducted on a 2D platform on which the properties of the nanoscale features such as spacing, width and height of ridges are constructed. The laser forms different features that mimic natural extracellular matrix features, which causes the cells to interact with the artificial construct as they would *in vivo*. Using laser-machined biomaterials with nanoscale features may potentially help us to gain a better understanding of biological mechanisms, such as cell adhesion on a biomaterial surface, which is mainly directed by molecular interactions at the nanoscale [59]. These studies contribute greatly to the fundamental understanding of the role of nanoscale topography, but have little correlation with the role of spatial nanostructures on 3D tissues. One of the main reasons this area is not progressing as fast is the lack of adequate methods to generate 3D nanostructures. Additive manufacturing technology is a group of techniques that could possibly address this, as it is able to fabricate 3D constructs based on a layer-by-layer principle.

The advantages of using the additive manufacturing approach to fabricate 3D nanostructures is the controllability of process parameters and, hence, the resulting consistency of scaffold properties. Selective laser sintering [60,61] and stereolithography [62,63] are two widely used techniques to fabricate 3D scaffolds for tissue engineering and regenerative medicine applications. However, distinct disadvantages limit their application. One of the obvious drawbacks is resolution, or rather the lack of resolution, as selective laser sintering and stereolithography can only fabricate precisely controlled scaffolds with geometrical dimensions ranging from tens to hundreds of micrometers, which is too large to mimic the unique microenvironment of natural tissues *in vivo* with submicron and nanoscale cues. With recent advancements in 3D laser nonlinear lithographic technology [64,65], multiphoton polymerization, especially two-photon polymerization (2PP), has been applied to create 3D nanostructures in a scaffold [66,67]. This technique has achieved the highest resolution (with feature sizes as small as 100 nm and even a size of 30 nm has been reported [68]) so far in the family of additive manufacturing technology. The resolution of 2PP is adjustable, which conveniences the tuning and thus saves fabrication time [69].

2PP has been applied to a wide range of materials, from synthetic polymers (e.g., biodegradable triblock copolymer [70] and nonbiodegradable polymer Ormocer®, VOCO GmbH, Cuxhaven, Germany [71,72]) to proteins (e.g., fibrinogen [73,74], collagen type I and bovine serum albumin [75]), and even different metal-based sol–gel composites (e.g., Zr- or Ti-based composites [76,77]). Among these widely used materials, PEGDA, with its biocompatibility and nonfouling properties, is a very good candidate for tissue engineering scaffold fabrication after 2PP treatment [78,79]. Figure 4 shows a scaffold fabricated by 2PP. The 3D structure is sophisticated and intricate, with a minimum feature size of 200 nm. Many PEGDA-based 3D scaffolds formed by 2PP have already been evaluated for their biocompatibility, including cytotoxicity [78], cell adhesion and cell viability [80]. There is a report that even showed a promising approach through the integration 2PP and laser-induced forward transfer to fabricate arbitrary PEGDA-based 3D structures with predesigned submicron features [81]. This technique offers a new approach to achieve 3D multicellular tissue constructs with an engineered extracellular matrix. It is also very interesting to notice that 2PP can crosslink natural polymers, potentially allowing the exploration of proteins and DNA as templates for the construction of 3D scaffolds [82]. In their report, the authors found that the laser formed protein scaffolds with precisely designed topographies that could be used as a new bioelectronics platform for monitoring and simulating biological processes [82], such as cellular signal transduction and neuronal networking [83,84]. Many properties of 2PP-generated 3D structures can also contribute to medical devices, such as small prosthetics [71,85,86]. Ovsianikov and coworkers manufactured total ossicular replacement prostheses out of Ormocer [87]. 2PP is a very important process in the synthesis

of Ormocer. The flexibility of 2PP makes the dimension of total ossicular replacement prostheses adjustable, which would be conducive to regenerative medicine applications.

Although 2PP has already proven to be a powerful technique for tissue engineering scaffold fabrication, there are still some drawbacks that limit its widespread usage. One of these factors is production time. Recently, several advanced methods have been explored to improve the throughput of laser fabrication. In a study by Zhang and Chen, the combination of 2PP and nanoimprinting was presented as an effective way to produce nanofeatures in the hydrogel in a massively parallel way [67]. Another trial utilized multibeam fabrication to shorten the 2PP process [88].

Using a laser to create nanofeatures is proven for 2D structures, but for 3D structures there remain challenges. However, there are emerging techniques that have shown feasibility, and as science and technology advances, this is probably going to be a very viable method.

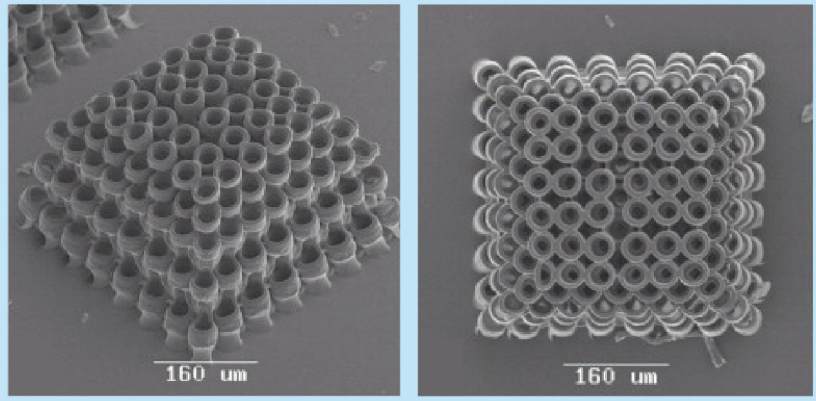

Figure 4. Highly organized 3D scaffold structure fabricated by two-photon polymerization. The minimum feature size is 200 nm.
Reproduced with permission from [78].

Bioprinting of cells

All the techniques discussed above involve the fabrication of advanced scaffolds to support cells.

In this section, another method to directly manipulate the microstructure of tissues at the cellular level to build up the organization of tissue, without the use of scaffolds, will be discussed. Bioprinting refers to a special additive manufacturing technology that processes cells and biological materials into the physical counterpart of a predefined 3D computer model. From the point of view of manufacturing, the resolution of the bioprinting process is below 100 µm, although not within the nanoscale. However, from the point of view of interactions between cells and materials, the scale of bioprinted biologics ranges from micrometers (e.g., cells) to nanometers (e.g., focal adhesion complexes and integrins).

In one study of bioprinting, cells were first prepared in the form of tissue spheroids in a robotic system [89], and then mixed with a hydrogel and printed one-by-one in a defined layout, such as a ring or a branched structure. Over time, these printed tissue spheroids can fuse and integrate to form tissue with an ordered organization [90]. In another method, a laser was used to assist printing of controlled 2D cellular patterns, such as the Olympic symbol shown in FIGURE 5, in a high-resolution and high-speed manner at the microscale [91]. Recently, multiple cell types have been separately mixed with crosslinkers ($CaCl_2$) and loaded into separate ink cartridges for inkjet printing [92]. The multiple-cell pie configuration shown in FIGURE 6A consists of human amniotic fluid-derived stem cells, canine smooth muscle cells and bovine aortic endothelial cells. All printed cell types maintained their viability and normal physiological functions within

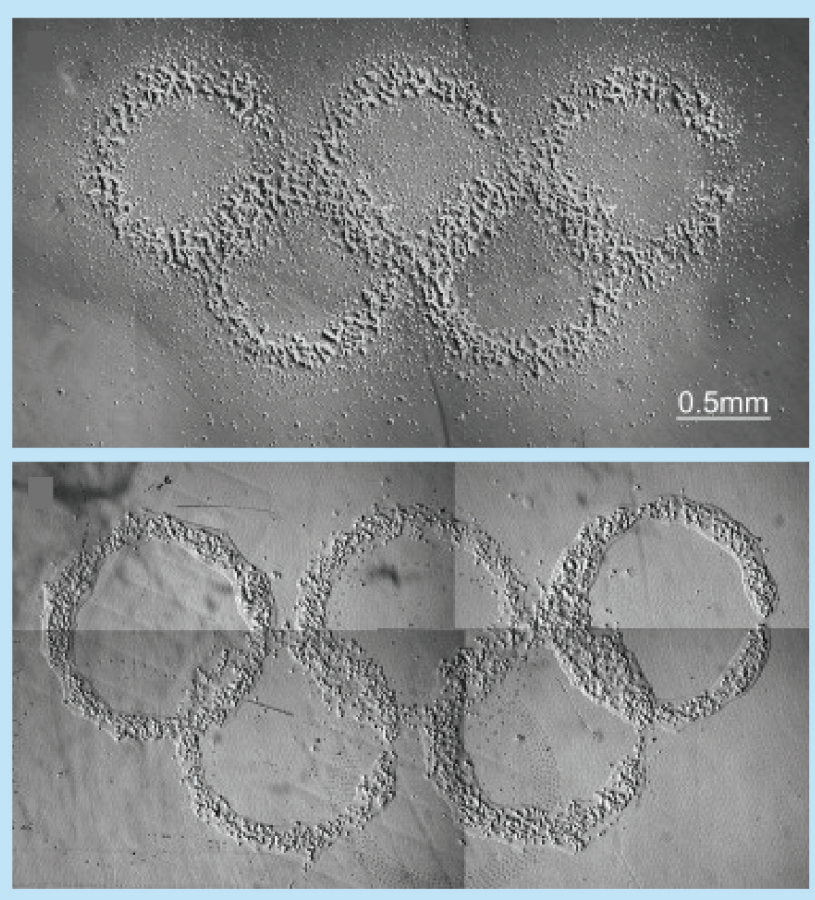

Figure 5. Laser-assisted bioprinting of 2D cellular patterns.
Reproduced with permission from [91].

the hybrid constructs (FIGURE 6B). The bioprinted constructs were adequately vascularized *in vivo* and matured into functional tissues (FIGURE 6C).

Bioprinting of cells is very much in its infancy and there are practical challenges ahead. Currently, one practical limitation of bioprinting is the weak mechanical strength of bioprinted hydrogels [93]. Development of a bioprintable hydrogel that is suitable for the bioprinting process, as well as for cell encapsulation and viability, is critical. Censi *et al.* evaluated the suitability of a biodegradable, photopolymerizable and thermosensitive A–B–A triblock copolymer hydrogel, in which poly(*N*-(2-hydroxypropyl) methacrylamide lactate) forms A blocks and hydrophilic poly(ethylene glycol) forms B blocks [94]. They demonstrated layer-by-layer deposition of hydrogel fibers, forming stable 3D constructs with high viability of encapsulated chondrocytes. Another practical challenge in bioprinting is the concurrent printing and culture of mixed multiple cell types. There are a few approaches that may be considered for fabricating a construct with mixed multiple cell types; for example, deposition of multiple types of cells through multiple nozzles or deposition of tissue spheroids that already contain a mixture of multiple cell types. Nonetheless, these approaches only address the issue of how to aggregate multiple types of cells; at the fundamental level, how to concurrently culture and grow multiple cell types is still unclear. Norotte *et al.* reported the use of various vascular cell types, including smooth muscle cells and fibroblasts, for bioprinting [2], but these cell types were not seeded at precise locations within a single scaffold and post-printing culture has not involved in their study. Although Schuurman *et al.* claimed to be able to bioprint a hybrid tissue construct [95], their actual work is limited to multiple cells of a single cell type, not multiple cell types. Currently, there is also considerable interest in the strategy of *in situ* bioprinting [96], in which the mixture of cells and hydrogels are directly deposited onto defect areas such as skin burns. This strategy could potentially eliminate the problems of *in vitro* bioprinting and provide rapid tissue repair, thus promising to be a new therapy in the future.

Conclusion

In conclusion, various nanobiomaterial strategies have shown some promising aspects in terms of

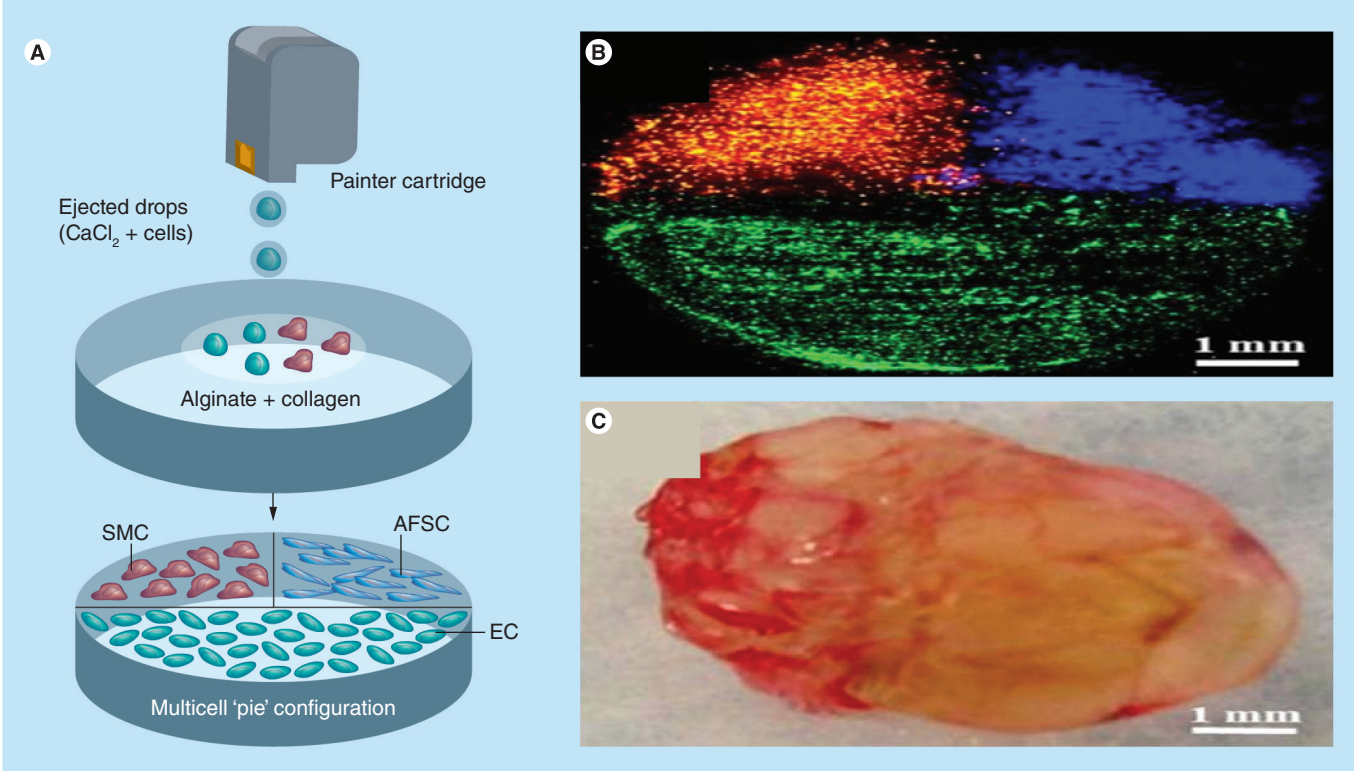

Figure 6. Inkjet bioprinting of 3D tissue engineering constructs consisting of multiple cell types. (A–C) The multiple-cell pie configuration. **(B)** All cell types maintained their viability and normal physiological functions within the hybrid constructs, **(C)** which were adequately vascularized *in vivo* and matured into functional tissues.
AFSC: Amniotic fluid-derived stem cell; EC: Endothelial cell; SMC: Smooth muscle cell.
Reproduced with permission from [92].

Table 2. Nanobiomaterial strategies for developing 3D organized tissue engineering constructs.

Nanobiomaterial strategy	Advantages	Disadvantages	Organization
Nanofibrous scaffolds and nanocomposites	Established 2D nanofeature guidance on cells and 3D bulk nanofibrous constructs available	Unorganized 3D bulk structure, inadequate pore size and strength, and poor consistency	Unorganized
Hydrogels	Ability to hold water and swell, resemble living tissues and ease of applicability	Inadequate bioactivity and strength, and poor internal structure	Less organized cell–scaffold constructs
Laser fabrication	Rapid fabrication and highly controllable organized 3D scaffolds available	Few biomaterials can easily be laser processed	Organized scaffold structure
Bioprinting	Established principle of layer-by-layer printing of preliminary cell/tissue constructs	Inadequate hydrogel strength, and great biological challenge of printing and culture of heterogeneous cells	Organized cells and tissue structure

Nanobiomaterial strategies are listed in ascending degree of organization.

addressing the issue of complexity and organization in tissues and organs, but no single strategy is a complete solution to this challenge. TABLE 2 summarizes the advantages and disadvantages of each and suggests that, based on the degree of organization of current 3D constructs, the bioprinting strategy is now closer to these aims than other strategies, and could be a viable approach in the future.

Future perspective

Various recent nanobiomaterial strategies have been reviewed in this paper to highlight their potential for engineering 3D organized tissue. Moving forward, tissue engineering and regenerative medicine will have to address the issues of complexity and organization in tissues and organs. Future work should include manipulation of nanobiomaterials toward the engineering of a more ordered 3D tissue microstructure, and should reveal more on the relationship between tissue microstructure and the resulting characteristics of an engineered tissue.

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Executive summary

- Tissue engineering and regenerative medicine are restricted by a limited tissue thickness and poorly organized tissue microstructures.
- In recent years, research on nanobiomaterials for tissue engineering and regenerative medicine applications has emerged, due to their ability to direct cell behaviors toward desired tissue outcomes.
- Recent progresses in 3D nanofibrous scaffolds and nanocomposites, hydrogels, laser-fabricated nano- and micro-structures, and bioprinting enable the possibility of developing 3D organized tissue engineering constructs to address the issues of complexity and organization in tissues and organs.
- Bioprinting is a very promising approach for developing 3D organized tissue constructs, but it is still in its infancy and is yet to overcome the practical challenges to truly deliver a printed functional tissue or organ.

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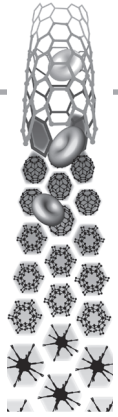

Laser-assisted cell printing: principle, physical parameters versus cell fate and perspectives in tissue engineering

We describe the physical parameters involved in laser-assisted cell printing and present evidence that this technology is coming of age. Finally we discuss how this high-throughput, high-resolution technique may help in reproducing local cell microenvironments, and thereby create functional tissue-engineered 3D constructs.

KEYWORDS: bioprinting ▪ cells ▪ modeling ▪ rapid prototyping ▪ tissue engineering

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On tissue engineering approaches

The loss or failure of an organ or tissue is one of the most frequent, devastating and costly problems in healthcare [1]. Current treatment modalities include transplantation of organs, surgical reconstruction, use of mechanical devices or supplementation of metabolic products. Epidemiological studies highlight tissue/organ shortage, which justifies original approaches to fulfill clinical needs [2]. Tissue engineering (TE) aims at providing regenerative medicine with original products. In addition, TE may provide original models (2D/3D) for fundamental research in biology [3]. Since the late 1980s and the creation of the first workable definition of hybrid artificial organs [4], an increasing number of research groups throughout the world have developed TE-orientated approaches. As stated by Langer and Vacanti [5], these approaches apply the principles of engineering and life sciences to the development of biological substitutes that restore, maintain or improve tissue or whole organ function. Generating biological tissues *in vitro* involves the use of engineering and material methods, the appropriate combination of cells and the suitable biochemical and physicochemical factors to mimic both the microenvironment of cells and the microarchitecture of tissues in the body. Traditionally, TE approaches use porous biomaterial scaffolds seeded with isolated autologous cells from the patient, culturing the constructs in a bioreactor and implanting the resulting cell/biomaterial complex back into the patient. With an appropriate scaffold that mimics the biological extracellular matrix, it is expected that the developing tissue will adopt both the form and function of the desired organ. Tremendous progress in the synthesis and manufacturing of biomaterials in order to obtain highly biocompatible and functional scaffolds has been made.

Tissue engineering has already proven its ability to move from the bench to the bedside [6]. For instance, blood vessel-, skin-, cornea- and bladder-related TE products have been successfully translated from research to clinical practice [7–11].

Despite these scientific progresses and clinical outcomes, engineered tissues and especially thick or complex tissues still suffer from reoccurring drawbacks:

- Cell penetration and adhesion is not very effective. One or several months may be required for the cells to adhere and proliferate into the scaffold. As a result, an incomplete colonization, limited to the scaffold's external layers, may occur [12];
- Organs and tissues are generally complex, and host different cell types. Cell-to-cell contact and cell-to-substrate interaction is critically involved in tissue morphogenesis and regulation, or healing. Consequently the need to promote cell-to-cell communication remains a very challenging issue for this kind of approach;
- The absence of built-in vascularization [12,13]. Most tissues in the body rely on blood vessels to supply individual cells with nutrients and oxygen. For a tissue thickness beyond 100–200 µm (the diffusion limit of oxygen), new blood-vessel formation is required, and this holds true for TE constructs [14].

The limitations of the scaffold-based TE lie in the initial paradigm of creating a functional 3D tissue structure by seeding cells (differentiated or stem cells) onto the scaffold, without any *ab initio* patterns. In other words, the question

future medicine part of fsg

of "what 3D pattern of cells and biomaterials must be fabricated?" has been consistently either eluded to or ignored, probably because no relevant technology has previously existed. This question, which is similar to Rivron's "How to orchestrate developmental mechanisms *in vitro*?" [15] is now a leading concern and should become the focus of studies in the future.

In this report we discuss the need to develop tools dealing with tissue complexity and anisotropy, and physical parameters involved in laser-assisted cell printing. We then present evidence that this technology is coming of age. Finally we discuss how high-throughput, high-resolution techniques may address these questions in the near future.

From macroscopic to microscopic TE

Stem cell fate is influenced by a number of factors and interactions that require robust control for safe and effective regeneration of functional tissue [16]. Coordinated interactions with soluble factors, other cell types and extracellular matrices define a local biochemical and mechanical microenvironment with complex and dynamic regulation that stem cells sense [15]. On a local scale, tissue development is, in part, regulated by the spatial and temporal distribution of cues (i.e., local gradients of soluble or insoluble factors, local physical forces). This suggests a dynamic interaction between form and function [17,18] and emphasizes the importance of shaping adequate multicellular, multifunctional geometries to promote proper tissue integration.

Tissue engineering approaches can be divided into three strategies based on the scale of spatial organization. First, macrosopic strategy can be likened to traditional TE in which cells are seeded onto a macroporous scaffold. Cells are expected to colonize the inner volume of the scaffold by cell mobility and proliferation, and fluid flow. As described above, advances in the design of smart scaffolds and in the understanding of tissue maturation within bioreactor chambers have produced functional tissues [19]. However, smart scaffolds do not present the ability to mimic the functional multicellular anisotropy of the host tissue. Second, mesostructures are based on cells' ability to self-assemble and their capacity to maintain viability and function when located within the diffusion limit of nutrient supply. These modular blocks, also termed organoids, can be fabricated *in vitro* using replica molding [20,21] or by shaping multicellular spheroids [22]. The modular approach enable the production of 3D modules in a variety of shapes (e.g., cylinders) with a lateral diameter between 40 and 1000 µm and cell densities of 10^5–10^8 cells/cm², and to allow fabrication of multicellular constructs (e.g., bone-mimicking construct including both osteoblasts, osteoclasts and endothelial cells). Finally, reproducing the local cell microenvironment can be thought of as the ultimate target for TE and cell patterning. Conceptually, it could be defined as the capacity of positioning a single cell into its most suitable environment. Coordinated interactions between soluble factors, different cell types and extracellular matrices (i.e., mechanical and biochemical cues) should be taken into account. Such a cell niche manufacturing approach is unique in its purpose of dealing with tissue complexity and engineering a desired tissue from the bottom up. A scaffold-free, bottom-up approach to TE has been proposed [23]. Interestingly, Albrecht *et al.* have presented a method for the rapid formation of reproducible, high-resolution 3D cellular structures within a photopolymerizable hydrogel using dielectrophoretic forces [24]. This technology allows production of microscale cell organization. However, deposition of extracellular material can not be geometrically controlled using this approach.

To address some of the aforementioned issues, some authors have suggested building 3D biological structures using bioprinting: the precise computer-aided robotic deposit of living and nonliving biomaterials with the purpose of bioengineering 2D cellular patterns and 3D tissue constructs [25]. Commercially available ink-jet printers have been successfully employed [26,27] to pattern biological assemblies according to a computer-aided design template (blueprint). Pressure-operated mechanical extruders have also been developed to handle cells and cell aggregates [28]. Laser-assisted printing has emerged as an alternative technology, which has the ability to overcome some of the limitations of ink-jet and micro-pen printing devices, namely, the clogging (due to viscosity, cell agglomeration or ink drying) of print heads or capillaries used by these printers to achieve micron-scale resolution. In addition to laser-guided direct writing, which is a technique capable of trapping multiple cells in a laser beam and depositing them as a steady stream on arbitrary nonabsorbing surfaces [29], laser-assisted bioprinting (LAB) has been developed. Based on the laser-induced forward transfer (LIFT) process, LAB is potentially more efficient than laser-guided direct writing for cell patterning and TE; optical trapping allows manipulation of individual cells or cell aggregates in a matter of seconds, while LAB can manage virtually single cell deposit at a rate of kHz.

Mechanism of laser-assisted bioprinting

A typical LAB set-up is generally composed of three elements: a pulsed laser source, a target coated with the material to be printed (the ribbon) and a receiving substrate (Figure 1). The ribbon is a multilayer component: a support, which is transparent to the laser radiation wavelength, is coated with a transfer layer, named bioink, composed of the heat sensitive biological material to be printed (e.g., biomaterials, cells, biomolecules). Depending on the optical properties of the bioink or laser wavelength, a laser-absorbing interlayer is necessary to induce transfer and is placed between the support and the bioink; hence, the term biological laser printing [30] or absorbing-film assisted-LIFT [31] is preferred to matrix-assisted pulse laser evaporation-direct write (MAPLE-DW) [32], which implies the vaporization of the first molecular layers of the liquid. Such an interlayer eliminates direct interaction between the laser beam and the bioink, while 99% of the nonreflected incident beam may be transmitted in the case of MAPLE-DW. This interlayer consists of a thin film (tens of nm) of metal (Au, Ti, Ag), metal oxide (TiO_2) or photo-decomposing volatile polymer (triazene). Even if the LAB printing mechanism depends on many parameters (Figure 1), it was found that the volume of deposited material depends linearly on the laser pulse energy, and that a minimum threshold energy has to be overcome for microdroplet ejection to occur [33,34]. The technology behind these techniques has been increasingly refined in recent years giving new highlights to the three regimes which are typically conserved experimentally: subthreshold, jetting and plume regimes [35]. LAB can be described by the following sequence of events.

Laser energy deposit (1 J/cm²)

Pulsed-energy deposition (typically 1–20 µJ per pulse) can be performed by means of nano second lasers with UV wavelengths (i.e., excimer lasers 193 nm, 248 nm or triple- or quadruple-frequency neodymium-doped yttrium aluminium garnet lasers (266 and 355 nm, respectively) or else with near IR wavelength (1064 nm). The laser energy-absorbing material at the bioink–support interface (as in MAPLE-DW) or of/near the absorbing interlayer (as in biological laser printer) rapidly evaporates (including normal boiling and phase explosion) upon the absorption of laser pulse energy and further plasma formation, and may further form a vapor bubble that expands towards the free surface.

Vapor bubble growth & collapsing (1 µs)

Bubble growth and collapsing are critical phenomena since they govern the printing regime and because they are related to the intensity of mechanical stress applied to cells within the bioink (see below). In addition to time-resolved imaging, which provided time scale and morphological information [35,36], bubble dynamics have been modeled analytically (using the Rayleigh–Plesset equation) and

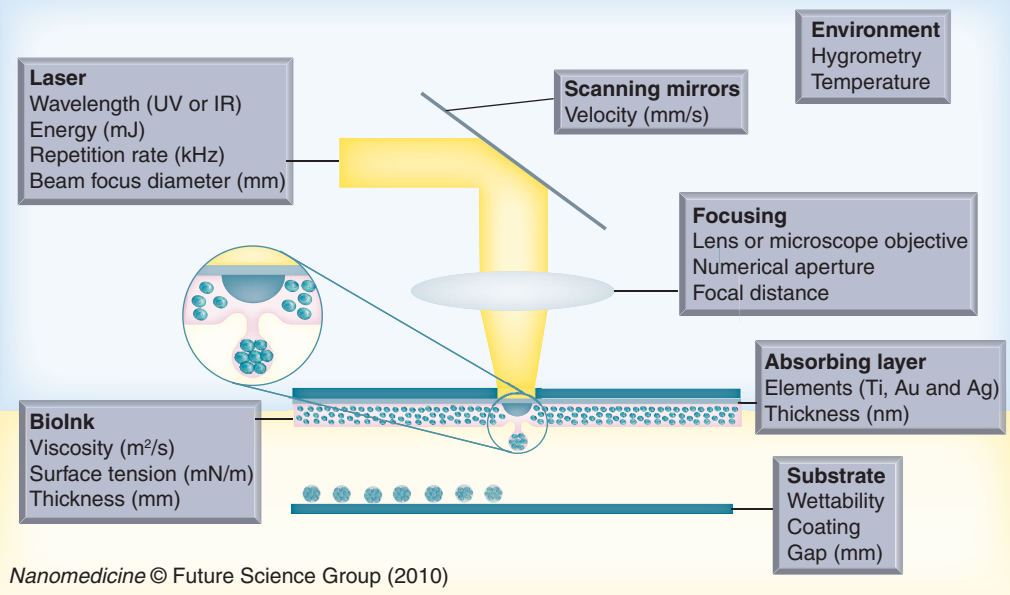


Figure 1. Laser-assisted bioprinting, featuring all the parameters involved in the process.

numerically [37,38] by including the effect of different medium material properties (e.g., viscosity and surface tension).

$$RR+\frac{3}{2}R^2=\frac{\kappa P\iota}{\rho\iota}\left(\frac{R_0}{R}\right)^{3y} - \frac{P\iota - P\upsilon}{\rho\iota} - 4\upsilon\frac{R}{R} - \frac{2\sigma\iota}{\rho\iota R}$$

As a result, it was demonstrated that, depending on the bioink compressibility, the bubble front rapidly reaches its maximum velocity (up to 100 m/s, 100 ns after plasma-induced generation) while the maximum bubble radius R_{max} (meaning when collapsing starts) was reached later (1.2 µs) [34]. These values were shown to diminish by increasing medium viscosity while bubble dynamics was shown to be insensitive to surface tension.

Interaction of the vapor bubble with the free surface

Since the size of the vapor bubble is negligible compared with bioink thickness, the bubble interacts with the free surface, and hence surface tension has to be taken into account. In this regard, it has been demonstrated (for standoff conditions) that when the bubble reaches R_{max}, it begins to collapse due to a high pressure region generated in the bubble apex, and a jet may be formed according to the dimensionless distance Γ, which is the ratio between the distance, h (distance between the initial vapor bubble centroid and the free surface), and R_{max} [39,40].

$$\Gamma = \frac{h}{R_{\max}}$$

Consequently, the three above-mentioned regimes do not solely result from laser energy (E) intensity but also from rheological properties (e.g., viscosity [υ], surface tension [σ]) and film thickness (ε) of the bioink. In other words, jetting does not simply occur on the basis of an energy threshold mechanism [33] but rather on the basis of a complex Γ (E,υ,ε,σ) threshold mechanism. Over a given laser energy for which a vapor bubble is formed at the absorbing layer–bioink interface, the three above-mentioned regimes can be described (Figure 2):

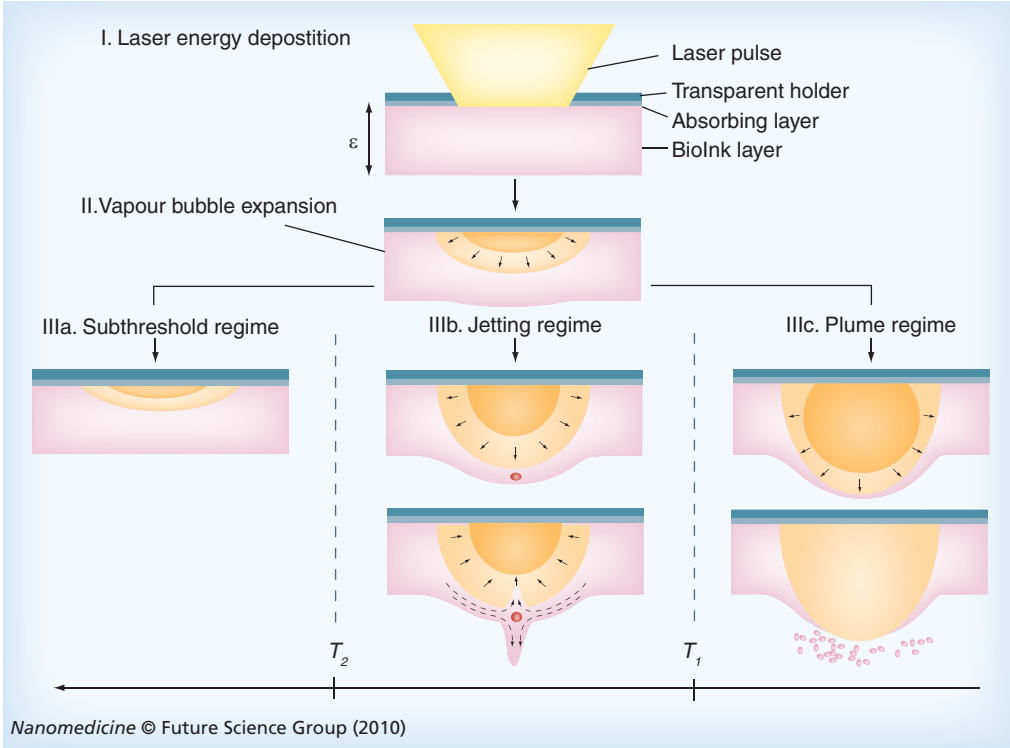


Figure 2. Mechanism for laser-induced droplet ejection. A vapor bubble is generated (see II) by vaporization of the absorbing layer and/or the first molecular layers of the liquid film. At given bioink viscosity and film thickness, jetting (see III.b) is observed for intermediary values of laser fluences ($\Gamma_1 < \Gamma < \Gamma_2$). For a lower fluence ($\Gamma > \Gamma_2$), the bubble collapses far from the free surface without generating a jet (see III.a). For a higher fluence ($\Gamma < \Gamma_1$), the bubble bursts to the surface, generating sub-micrometer droplets (see III.c). Increasing film thickness or bioink viscosity leads to increased threshold Γ values.

Table 1. Key milestones in the development of safe laser-assisted bioprinting procedures.

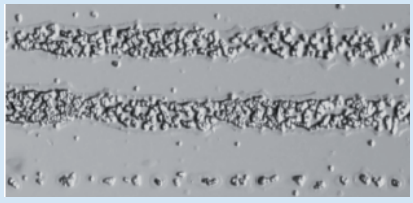
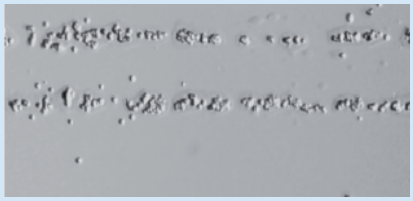
Laser-assisted cell printing method	Key milestones	Ref.
MAPLE-DW, 193 nm	Laser transfer by MAPLE-DW does not damage printed protein epitopes.	[52]
MAPLE-DW, 193 nm, 20 ns, 100 Hz	Transfer technique was found to be compatible with cell transfer as all transferred cells were found to be viable.	[32]
MAPLE-DW, 193 nm, 30 ns	When pluripotent embryonal carcinoma cells (P19) were printed onto a thick layer of Matrigel™ (> 40 µm), more than 90% of the cells survived the transfer process, remained viable and could differentiate into the neural or muscle cell lineage.	[46]
BioLP, 193 nm, 20 ns	A laser absorbing interlayer was placed between the incident laser pulse and biomaterials to be printed. Proof-of-principle experiments of multicolor printing and 3D cell pattern using BioLP (by spreading a 75 µm thick layer of Matrigel between each cell layer).	[30]
BioLP, 248 nm, 2 ns, 100 Hz	Number of cells per spot was shown to be determined by random sampling statistics. Demonstration of minimal expression of heat shock proteins by printed cells.	[53]
AFA-LIFT, 248 nm, 30 ns	Using time-resolved imaging, cell printing was shown to occur on a 1 µs time scale at a constant velocity with an estimated acceleration of 10^7 m/s^2.	[31]
MAPLE-DW, 193 nm, 10 Hz	Co-deposition of hydroxyapatite, MG 63 osteoblast-like cells and extracellular matrix demonstrated the possibility to print complex bioinks.	[54]
MAPLE-DW, 193 nm	Formation of a 3D neural network by transferring B35 neuronal cells to different depths in Matrigel by applying increasing laser fluences (energy per surface; J/m^2).	[55]
MAPLE-DW, 193 nm, 30 ns, 10 Hz, triazene layer	An intermediate layer of absorbing triazene polymer (dynamic release layer) was used to provide a gentler and more efficient printing (i.e., at lower fluences than usual) of B35 neuroblast cells.	[56]
LIFT, 800 nm, 120 fs, 1 kHz	Gap between NIH3T3 fibroblasts containing ribbon and substrate was filled by culture medium to avoid cell stress due to film drying.	[57]
MAPLE-DW, 355 nm, 15 ns	Mammalian embryonic stem cells were printed using a thick polyimide absorbing layer, which differs from a dynamic release layer.	[58]
BioLP, 266 nm, 5 ns, TiO_2 (40 nm)	Glycerol was substituted with methyl cellulose to avoid ink dehydration without toxic effects.	[42]
MAPLE-DW, 93 nm, 300 Hz, triazene DRL	Single-cell printing and groups of cells (approximately 20–40 cells) were obtained when the ribbon was preliminary seeded with a lower cell concentration and near 100% confluence, respectively.	[59]
MAPLE-DW, 193 nm, 2 ns	Some of the MAPLE-DW process-induced damage to yeast cells was reversible and the post-transfer yeast cell recovery was a function of the laser fluence (85–1500 mJ/cm^2)	[44]
Laser-assisted bioprinting, 1064 nm, 5–10 kHz	High-throughout cell printing (up to tens of thousands of droplets per second) was demonstrated using an original workstation. EA.hy926 endothelial cells remained viable after printing using a near-IR nano second laser.	[43]

BioLP: Biological laser printe; DRL: Dynamic release layer; LIFT: Laser-induced forward transfer; MAPLE-DW: Matrix-assisted pulse laser evaporation-direct write.

- If Γ is higher than a threshold value Γ_2 the droplet ejection cannot occur since the bubble expansion is too weak to reach the free surface much; see III.a in Figure 2). This is termed the subthreshold scenario, in which no material can be transferred except if the substrate is in close proximity with the ribbon;
- When Γ is lower than a threshold value Γ_1 the bubble expansion is so violent that it overcomes surface tension resulting in bubble bursting, and hence, liquid splashing onto the substrate; see III.c in Figure 2). This is therefore termed the plume scenario
- If Γ is between Γ_1 and Γ_2: the bubble expands, then collapses and finally a jet is formed; see III.b in Figure 2). Termed the jetting scenario.

Jetting (50 m/s)

By using time-resolved imaging, Duocastella *et al.* recently demonstrate that a long and uniform jet is developed, which advances at a constant velocity (20–150 m/s, depending on experimental conditions) until it reaches the receptor substrate [41]. For the lowest fluences leading to jet formation (e.g., $\Gamma \rightarrow \Gamma_2$), the jet may recoil before reaching the substrate. However, reduction of the gap distance could lead to material deposition onto the receptor substrate. At higher fluences ($\Gamma \rightarrow \Gamma_1$), the jet inertia is high enough to surpass the recoiling force exerted by the surface tension and elasticity of the ink. When a jet has reached a certain length, it becomes unstable and finally breaks due to surface tension effects in the so-called Rayleigh–Plateau instability.

Diaphragm aperture stop (mm)	Laser energy (μJ)
18	
11	16
5	
18	
11	8
5	

Figure 3. Cell printing resolution according to the diaphragm aperture stop (mm) and the laser energy deposit (μJ). The cell concentration of the ink (DMEM, 1% alginate (w/v), 5% glycerol) was 10^8 cell/ml. × 25 magnification.

Deposit: landing

Depending on substrate surface properties, the kinetic energy of the droplet/jet as well as bioink viscosity, droplets collected onto the substrate may exhibit different morphologies, which can be related to splashing and spreading phenomena. This has not been studied in LAB conditions, although it is an important issue in surface science. For moderate initial energies, the surface tension will be able to absorb the initial kinetic energy while for higher energies, the surface tension is not sufficient to stop the outward motion as the drop spreads upon impact, which induces formation of small satellite droplets. In addition, viscosity has been shown to minimize the splashing effect for a given condition of droplet landing onto one substrate [GUILLEMOT *ET AL.* UNPUBLISHED DATA].

Droplets as small as 8 μm have been experimentally produced by reducing the air gap distance and thus working in conditions close to the subthreshold scenario ($\Gamma \rightarrow \Gamma_2$) [33].

Considering safe & high resolution procedures using laser assisted cell printing

Laser-assisted cell printing has been performed in numerous studies over the last five years. Key milestones on the way to developing safe laser-assisted cell printing procedures are reported in TABLE 1. During this period of time, the potential damages caused by the above-mentioned printing steps have been addressed. Besides virus- or microorganism-induced injuries, cell integrity and cell fate might be altered by mechanical, thermal, chemical or/and biochemical stresses. Regarding the LAB process, and considering that sub-threshold and plume scenarios are not compatible with controllable and safe procedures, cell injury may be associated with: bioink composition, interactions of cellular components with light, pressure generated during bubble growth, shear stress within the jet and landing conditions.

Regarding chemical change, biocompatibility of the cell-containing matrices have been addressed with a focus on glycerol content, which is routinely added to avoid dehydration of the bioink caused by the unfavorable surface-to-volume ratio once it is spread onto the ribbon. To limit toxicity induced by glycerol, it has been replaced with methyl cellulose [42]. Also, high-throughput printing may be carried out to reduce evaporation [43]. Indeed, it was recently shown that high speed cell printing (1–100 kHz) is possible and dramatically shortens the printing process [GUILLOTIN B, SOUQUET A, CATROS S *ET AL.*: CELL PRINTING ASSISTED BY LASER DOES NOT PRESENT A COMPROMISE BETWEEN RESOLUTION AND SPEED. UNPUBLISHED DATA].

While calculations have shown that much of the laser energy passes completely through the liquid layer, raising the possibility that the UV laser light could damage the biological structures, it has recently been demonstrated that yeast cells do not suffer from UV irradiation when printed by MAPLE-DW [44]. Nevertheless, this issue remains relevant to *in vivo* printing when body tissues are irradiated for a longer duration [45].

Cell damage due to mechanical stress during laser-assisted cell printing has been observed and is an important issue for further innovations in TE. Mechanical stress can have many origins: increasing hydrodynamic pressure during bubble growth (it was estimated to be a few MPa at a certain distance from bubble front [37]) shear stress during jetting (which depends on jetting speed and thus Γ conditions); and landing conditions (which are governed by the initial jet velocity and the softness and thickness of the substrate). As described above, increasing bioink viscosity or alternatively, reducing laser energy, leads to both reduced bubble expansion velocity and jet front speed. Regarding landing conditions, Ringeisen *et al.* have shown that cell viability is increased to 95% when the substrate is coated by a 40-μm thick Matrigel™ layer [46]. By means of numerical modeling, this result was correlated by Wang *et al.* to a decrease of the first impact-induced stress (von Mises stress) from 3 MPa to 0.86 MPa (for a 50 m/s jet velocity), respectively,

while second impact-induced stress (onto a hard substrate) may also be observed when mattress thickness is less than 40 µm (0.94 MPa for 20-µm coating thickness) [47]. Alternatively or additionally to the landing mattress, a high viability level can also be obtained when a viscous bioink, such as an alginate solution is used [43]. Heat shock protein 60/70 expression has been studied as a potential marker of heat and shear stress sensed by the cells during the printing process [31]. Results show that heat shock protein 60/70 expression is not altered in printed cells compared with control cells. The absence of a detectable effect of LAB on the printed cells has been demonstrated [48]. These data suggest the printed cells may have been exposed to an elevated heat or shear stress for a period of time short enough (few µs) to keep damage below detection thesholds.

Nevertheless, even if safe cell-printing procedures are developed, further studies need to performed to rule out any cell damage, and to determine the effective ratio between the number of printed cells and the number of cells embedded into the ink that are crossed by the laser beam.

Regarding cell printing resolution, LAB sets the benchmark as it is able to print cells one by one, next to each other. Given a 10 µm diameter for a nonadherent cells, maximum cell printing resolution is 1000 cells per cm. Such a resolution has been achieved recently at a high printing speed (5 kHz) (Figure 3) [44]. Although optimization remains possible in terms of higher throughput and higher resolution, such printing resolution and speed are key requirements for cell microarray production or 3D construct fabrication.

Future perspective

In the near future, LAB should be refined thanks to numerical studies that have been recently undertaken [37,38]. Hence, transient values for heat and pressure should be determined and subsequently controlled through manipulation of experimental parameters (e.g., irradiation conditions). Moreover, while jetting conditions have been shown to be subject to a complex threshold mechanism involving energy, viscosity and film thickness, the effects of viscoelastic ink properties on jet formation and recoil should be addressed. Finally, printing conditions without a metallic interlayer should be developed to avoid the potential cytotoxic effect of its residues.

In our opinion, the main issues over the next 5–10 years concerns biological and developmental studies. Developing tools such as LAB would allow us to create and manipulate the *in vitro* cell microenvironment on demand by controlling intensity and shape of cell patterns and morphogen gradients [49,50]. Studies would also deal with generating artificial cell niches by co-depositing a suitable combination of stem cells with extracellular matrix components [51]. In relation to these issues, mechanical and topological cues should be studied using bottom-up approaches for engineering tissues. Combining LAB with other laser-assisted processes, such as machining and polymerization, should be addressed with specific attention on integrating these different processes in the same workstation to guarantee subcellular resolution. Finally, while cell chip fabrication using LAB can be envisaged, other original applications such as medical robotics should be developed in the coming years [46], allowing LAB workstations to leave physics laboratories for biological benches [52–59].

Executive summary

- Regenerative medicine is the process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage or congenital defects.
- Bioprinting consists of computer-aided robotic transfer of living and nonliving biomaterials with the purpose of bioengineering 2D cellular patterns and 3D tissue constructs.
- Laser-assisted bioprinting (LAB) is based on the laser-induced forward-transfer (LIFT) technique in which a pulsed laser is used to induce the transfer of material from a source film spread onto an optically transparent quartz support to a substrate in close proximity to or in contact with the film.
- LAB is a noncontact, nozzle free, high resolution and high speed bioprinting technique. It allows the deposition of volumes smaller than a picoliter, at a micrometer scale resolution and at a meter per second writing speed.
- Jetting conditions are subject a complex threshold mechanism involving laser pulse energy, surface tension and viscoelastic properties of the bioink, as well as the bioink film thickness.
- Printed cell viability and preservation of cellular function are strongly related to hydrodynamics, which govern cell landing conditions onto the substrate.
- Complex multicomponent and 3D printing have already been performed.
- In addition to *in vitro* printing of cells and biomaterials, *in vivo* LAB is also feasible, which offers new opportunities in the field of medical robotics.
- LAB could enable the on-demand creation and manipulation of *in vitro* cell microenvironment by controlling intensity and shape of cell patterns and morphogen gradients.

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Special Report

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Regenerative Medicine

Operating RegenMed: development of better in-theater strategies for handling tissue-engineered organs and tissues

Tissue engineering *ex vivo* and direct cellular application with bioscaffolds *in vivo* has allowed surgeons to restore and establish function throughout the human body. The evidence for regenerative surgery is growing, and consequently there is a need for the development of more advanced regenerative surgery facilities. Regenerative medicine in the surgical field is changing rapidly and this must be reflected in the design of any future operating suite. The theater environment needs to be highly adaptable to account for future significant advances within the field. Development of purpose built, combined operating suites and tissue-engineering laboratories will provide the facility for modern surgeons to treat patients with organ deficits, using bespoke, regenerated constructs without the need for immunosuppression.

Keywords: biotechnology • operating room • operating theater • reconstruction • regeneration • regenerative medicine • scaffold • stem cells • surgery • tissue engineering

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Background

Significant advances have been made in regenerative medicine ('RegenMed') over the past decade and the field is advancing at a rapid pace. RegenMed now permeates through every surgical specialty [1,2]. The combination of tissue engineering *ex vivo* and direct cellular application with bioscaffolds *in vivo* has allowed modern surgeons to restore and establish function throughout the human body from replacing airways, the GI tract, hepatobiliary system, myocardium, kidneys, urinary tract to the skin, bone and connective tissues [3–8].

Although a proportion of the literature surrounding regenerative procedures is based on preclinical laboratory work, the clinical evidence for regenerative surgery is undeniably growing, and coupled with this is the need for the development of more advanced regenerative surgery facilities [9]. Achieving these advances in surgical science has required highly qualified, large multidisciplinary teams and advanced tissue-engineering facilities.

Alongside the scientific trials have been the ongoing debates regarding the ethical and practical issues surrounding the field of stem cell and animal research [10]. As a consequence, this expanding area of surgery has been limited to quaternary centers with substantial funding and international expertise. The future of regenerative surgery will be the development of specific operating theaters that have the capacity for on-site tissue engineering, with stem cell acquisition, scaffold generation and direct graft implantation, all within reach of the multidisciplinary team performing the operation.

Methods

We conducted a PubMed and ClinicalTrials.gov review of the literature surrounding RegenMed, tissue engineering and their role in the surgical field using variants of the search terms 'regenerative medicine', 'regenerative surgery', 'stem cells', 'tissue engineering', 'tissue transplantation' and 'biotechnology' from database inception to 10 May 2014. We used Boolean operators to refine our search and included all articles regardless of date and article type in the initial search. Articles were analyzed and selected based on

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the relevance to the topic of interest and were excluded if they were not in English or were duplicate in material covered. Review articles were preferred to original papers in topics where there was more substantial literature, whereas primary research articles were preferred in more innovative areas. The references of relevant articles were analyzed for further material. Information was included from basic science research, completed early-phase clinical trials and on-going clinical trials (Figure 1).

Principles of RegenMed

RegenMed replaces or regenerates human cells, tissues and organs, to restore or establish normal function [11]. The regeneration of tissue can take place either *in vivo* or *in vitro*, and may utilize stem cells, natural or synthetic bioscaffolds and bioactive molecules to induce cell differentiation, proliferation and genetic manipulation. [2] In reality, most modern techniques involve a combination of techniques depending on the end goal of the regenerative procedure [12].

Cells

Autologous cells are used most often for regenerative purposes (because they are not rejected), although cells can be sourced from allogenic or xenogenic donors. Ideally, they must be nonimmunogenic, highly proliferative, easy to harvest and possess the ability to differentiate into a variety of cell types with specialized functions [13]. Acquisition of stem cells for clinical regenerative purposes can be from several sources and these may be totipotent, pluripotent or tissue-specific, including mesenchymal stem cells (MSCs) from bone marrow/adipose tissue/umbilical cord/placenta,

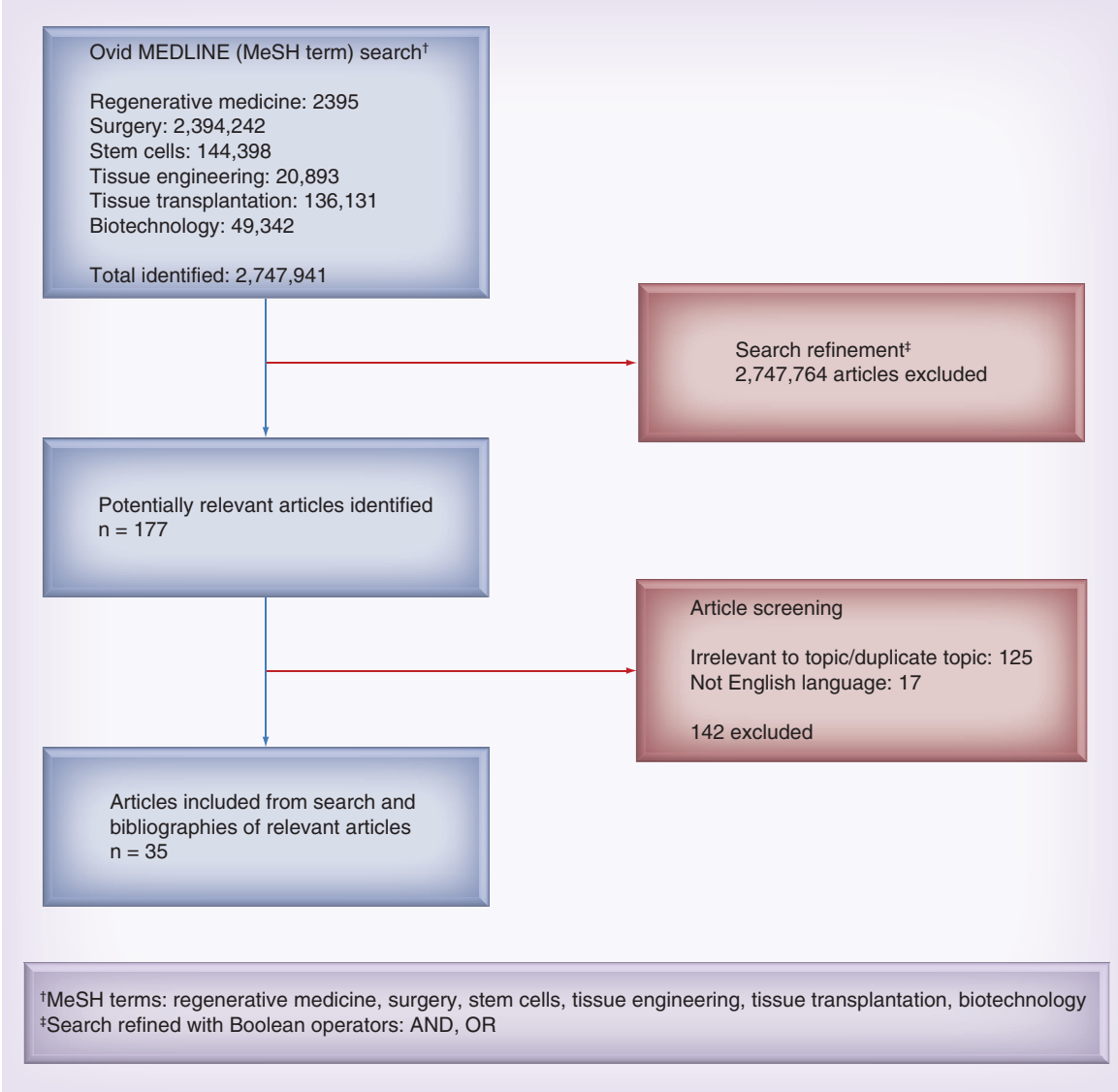

Figure 1. PRISMA flow chart depicting the search strategy that was employed in the generation of this review.

satellite cells, muscle stem cells, embryonic stem cells, induced pluripotent stem cells or amniotic fluid stem cells [14–16]. Many of these have already satisfied preclinical criteria and are being tested in clinical trials, or are in late preclinical phase. Hare and colleagues used MSCs to promote ventricular remodeling in patients with ischemic cardiomyopathy [17]. Cossu and colleagues are currently conducting a clinical trial of mesangioblast implantation for treatment of Duchenne's muscular dystrophy, while we and our colleagues are commencing a UK-based trial of tissue engineered laryngeal replacements for patients postlaryngectomy funded by the UK MRC (RegenVOX) [18,19].

Amniotic fluid stem cells have particular relevance in the prenatal generation of tissue for children who have been with congenital abnormalities *in utero*, allowing immediate graft implantation following birth [6]. The choice of specific stem cell to use depends on the tissue that is going to be replaced by the procedure; for example, whereas one cell type may be better for developing tissue for reconstruction of the trachea, a different cell is likely to be needed for constructing heart valves.

There is growing evidence that tissue-engineered cardiac muscle can be used to restore function following myocardial infarct in rats. In one study, tissue-engineered heart tissue, harvested from neonatal rat heart cells, prevented further dilation, induced systolic wall thickening of infarcted myocardial segments and improved fractional area shortening of infarcted hearts compared with controls [20].

Scaffolds

The prime requirements for any scaffold in tissue engineering are biocompatibility, nonimmunogenicity, the capacity to sustain and/or promote the growth of the relevant cells/tissue, and provision of a template for tissue growth in three dimensions. Many different materials (natural and synthetic, biodegradable and permanent) have been investigated. Synthetic materials offer strength, processability, degradation, microstructure and permeability, but can predispose to inflammation and foreign body reactions. The most commonly used synthetic scaffolds are polyglycolic acid structures, sometimes in combination with poly-4-hydroxybutyrate [21]. Natural scaffolds can be formed from the *in vivo* extracellular matrix components and, thus, have intrinsic interactive properties such as cell adhesiveness and enhanced biocompatibility [12,13]. However, natural materials are prone to attack from the host immune system, leading to inflammation and fibrosis and distortion of the form and function of the graft. Some of these limitations can be addressed using synthetic scaffolds. However, there are other challenges associated with synthetic scaffolds, such as designing optimal microstructures to support cell growth and achieving a balance between polymeric degradation and tissue formation [22].

Various different decellularization methods have been employed to date, including both physical and chemical methods [13]. The detergent–enzymatic decellularization method has made it possible to create a biocompatible acellular matrix, whereby cells can offer improved survivability, preserved structural integrity, biomechanical advantages, extracellular matrix stability, and better cell adhesion and differentiation, and has been shown to modulate both humoral and cell-mediated immune responses, thereby minimizing immune recognition and classical transplant rejection [22–26]. Thus, patients have received tissue-engineered airways from decellularized allogenic scaffolds and, to date, have not developed anti-HLA antibodies to date in the absence of immunosuppression [27–29]. However, the process of decellularization is not applicable to all tissues, most notably for the development of aortic valves, where biopolymers are more suitable.

Tissue generation

The first stage in the process of *in vitro* tissue generation to replace lost or inadequate tissue involves cell harvesting from a biological source containing stem cells, for example bone marrow. The stem cells should then be isolated from the sample and expanded in cell culture. Following this, the *in vitro* expansion of the stem cell population takes place in a bioreactor to stimulate proliferation and differentiation of specific cell populations, as well as adhesion to the scaffold. Most of the early work in tissue engineering used standard static cell-culture conditions for the *in vitro* fabrication of tissue before implantation. Since then, the introduction of bioreactors has enabled the *in vitro* culture of greater volumes of cells by producing a dynamic microenvironment culture system. Bioreactors, by flow and mixing, can ultimately facilitate cell integration and growth inside the scaffold or matrix by amplifying the mass transfer of nutrients, gases, metabolites, and regulatory molecules and also by providing mechanical stimulation that can induce specific cellular adaption [12–13,30]. Finally, once the autologous graft has been generated, it can be implanted into the patient to restore lost tissue or establish functionality.

Routine cardiac valve replacement currently relies on the use of mechanical valves with formal, lifelong anticoagulation or porcine tissue valves with limited longevity. Tissue-engineered valves have been generated using both synthetic and biological scaffolds and, by remodeling and regeneration, are able to overcome these limitations [31].

Macchiarini *et al.* successfully applied an *in vitro* tissue-engineering process using a donor trachea, which was decellularized and then readily colonized by the recipient's epithelial cells and chondrogenic MSCs [28]. Importantly, the nature of the patient's disease in this case made *in vitro* (thus delayed) preparation of the tissue-engineered organ possible with subsequent implantation.

For *in vivo* tissue generation, the initial cell harvesting, isolation and preparation are the same. The cells are then directly infiltrated onto a scaffold and *in vivo* cell seeding and adhesion takes place under the influence of cytokines and biochemical to promote differentiation [12]. In comparison to the work by Macchiarini and colleagues, Elliot and colleagues describe a case where the need for intervention was more immediate and, thus, an *in vivo* method was adopted. In this situation, a child required emergency implantation of a neotrachea. By using a decellularized cadaveric trachea seeded with autologous MSCs and exposed to topical biochemical inducers of differentiation, the airway was successfully reconstructed with total functionality at 2-years postoperation [27].

RegenMed operating theater: design & practicalities

Our concept of the future design and practicalities of creating an optimized operating suite for RegenMed is based on our prior experience of transplanting tissue-engineered constructs into experimental animals and children with unmet clinical needs [27]. Currently, Tengion Inc. (NC, USA) has been a pioneer in the regenerative surgery with engineered constructs. Their system involves the harvesting of stem cells in the hospital setting, with the cell-culture and tissue-engineering steps taking place off-site [32]. Although this system has been successful up to a point, the next stage will encompass the entire regenerative and reconstructive process being performed at one site. Alternatively, adipose-derived and bone marrow-derived MSCs can be procured intraoperatively (e.g., Celution® from Cytori Inc. [CA, USA], or MarrowXpress™ from Celling Bioscience [TX, USA]) and used as suspensions of mononuclear cells or in combination with scaffold materials, such as demineralized bone.

The future RegenMed Operating Suite will be equipped to harvest autologous stem cells from patients immediately prior to surgery. An example method for this would be to harvest a sample of bone marrow aspirate, or adipose tissue, from the patient. The suite should house a centrifuge, cell isolation apparatus and cell culture facilities that would allow the team to immediately isolate MSCs from the sample. Cells should be stored in optimal conditions (37°C, 5% CO_2) in an incubator, housed within the operating theater for *in vivo* tissue regeneration, or in the adjacent laboratory for seeding onto scaffolds for *in vitro* tissue regeneration. Sanyo™ (Osaka, Japan) have developed an integrated cell processing workstation that enables cell culture and manipulation in an efficient, aseptic and cost-effective way without the need for a dedicated clean room [33]. This kind of workstation could be integrated into the design of the RegenMed theater, either within the suite or immediately adjacent, allowing the team to harvest, culture and apply autologous stem cells to the bespoke scaffold within the same aseptic procedure. All facilities would be required to be compliant with EU GMP standards and, at present, to be licensed by the Medicines and Healthcare Products Regulatory Agency in the UK as a pharmaceutical manufacturing site. This is a major undertaking and will require substantial resources, which will exceed the costs of running a purpose-built RegenMed theater. This is a rapidly changing field and regulators around the world are already considering how operating theaters can become satellites of licensed GMP manufacturing laboratories (Figure 2).

It is likely that *in vivo* tissue engineering will be better suited to this type of set-up as it would prevent the need for multiple procedures, both respect to harvesting stem cells and transplanting the tissue-engineered construct. Alternatively using an *in vitro* approach, a dedicated RegenMed laboratory adjacent to the operating theater containing a bioreactor would be optimal so as to facilitate timely generation of the organ tissue and it is safe transfer to the patient at the time of surgery. The RegenMed theater itself would need to have facilities in place to store a variety of scaffold materials, in order to accommodate the vast variability in application and suitability of both natural and synthetic products. Ideally, the suite should also house apparatus to decellularize cellular matrices, such that bespoke scaffolds can be generated *ad hoc*.

Several bioactive agents are currently available for the generation of improved regenerated grafts. Granulocyte-colony stimulating factor has historically been used to stimulate proliferation of progenitor cells before bone marrow cell harvest and transplant, but has more recently been found to augment MSC recruitment into a bioscaffold [34]. Human recombinant erythropoietin may improve survival of cells in tissue that is oxygen depleted due to immature angiogenesis [35]. TGF-β has been shown to potentiate differentiation of MSCs into chondrocytes, but also promotes myofibroblast-mediated scar formation [27,36]. The RegenMed theater would have the facility to utilize a range of these biomolecules, ready to be applied to the tissue-engineered construct, thereby allowing a variety of tissues to be generated, depending on the requirements of the individual patient.

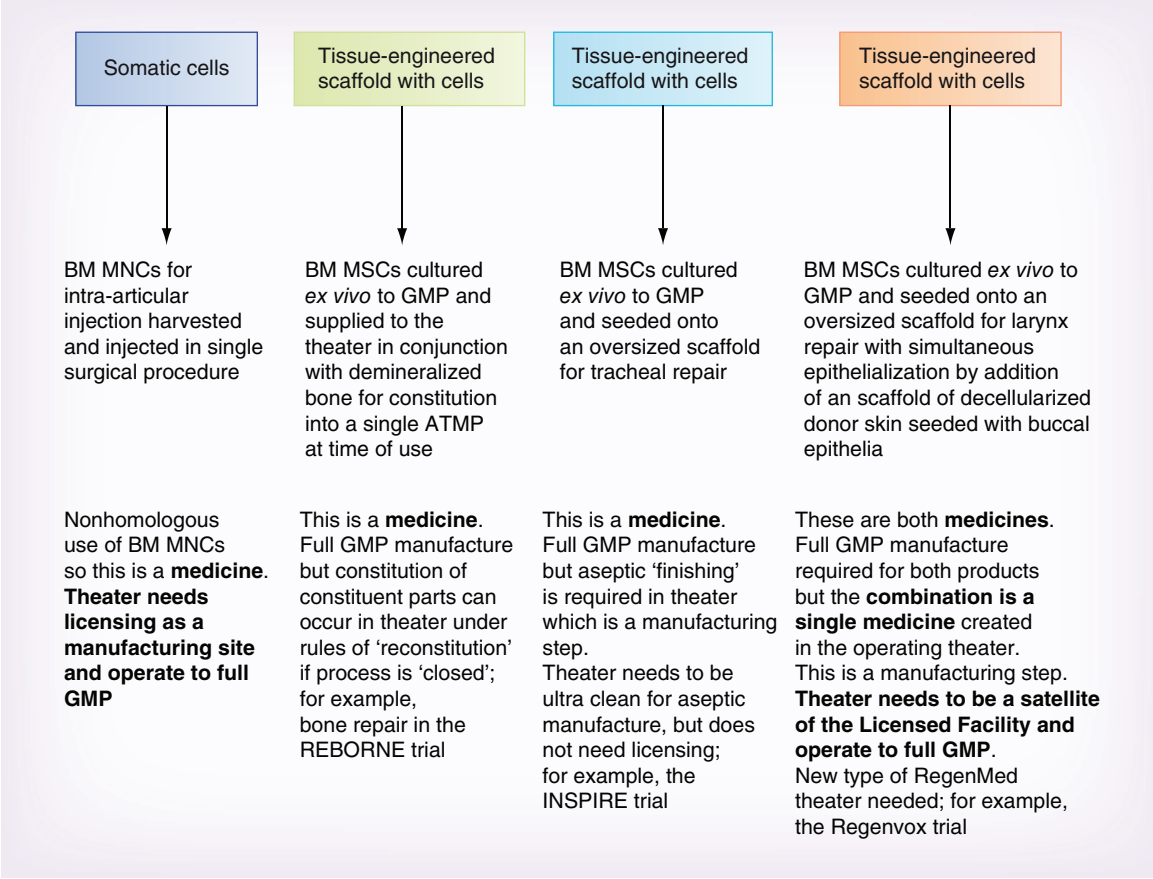

Figure 2. GMP compliance of various tissue-engineered therapies in clinical practice.
ATMP: Advanced therapy medicinal product; BM: Bone marrow; MNC: Mononuclear cell; MSC: Mesenchymal stem cell; RegenMed: Regenerative medicine.

Several other important aspects of the RegenMed theater need to be mentioned. First, is the ultraclean nature required for the aseptic processes – class 100 is required compared with conventional class 10,000 operating theaters (i.e., 100-times cleaner). Second, imaging equipment, with respect to monitoring of the graft during implantation requires: cell and tissue labeling with dyes and nanomarkers for super high-resolution CT and MRI, or bioluminescence akin to Luminex technology used in animal studies. Progress is required in this area. Third, the capacity for a multidisciplinary team working in a segmented theater design to maintain an aseptic cordon around the patient is required that would still allow highly specialized radiology and near-patient product finishing by GMP scientists within the theater complex. Open theaters with invisible air showers between multidisciplinary team groups could be one solution to overcome this.

Finally, the role of robotics and 3D imaging in the operating theater is an ever-growing area; its application is broad, but is predominantly utilized in urological and gynecological surgery. Although its role is distinct from that of RegenMed, they are both pushing the frontiers of modern surgery and for this reason, it would be prudent of the RegenMed operating theater to have adaptations that would allow surgical robotics to be easily integrated into its design, thereby leading to the creation of hybrid operating rooms capable of sustaining multiple technological advances in the forthcoming years [37–39].

Conclusion & future perspective

The field of RegenMed, as applied to surgical disease, is a rapidly changing field and this would need to be reflected in the design of any future operating suite with RegenMed in mind. The theater needs to be highly adaptable to account for significant advances that we can expect within the field over the next few years. There are still many unanswered questions, which will necessitate further laboratory-based research in the shorter term, with formal clinical trials in the longer term [40]. However, undeniably surgical intervention, in combination with the science of tissue engineering and RegenMed, can provide a definitive surgical cure for many conditions for which there is no current treatment strategy.

Current operating theaters are poorly equipped to deal with the scientific advances in tissue engineering. Development of purpose built, combined operating suites and tissue-engineering laboratories will provide the facility for the modern surgeon to treat patients with tissue or organ deficits, using bespoke, regenerated constructs without the need for immunosuppression.

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Executive summary

Premise

- Significant advances have been made in regenerative medicine (RegenMed) over the past decade and the field is advancing at a rapid pace.
- The combination of tissue engineering *ex vivo* and direct cellular application with bioscaffolds *in vivo* has allowed modern surgeons to restore and establish function throughout the human body.
- The evidence for regenerative surgery is undeniably growing, and coupled with this is the need for the development of more advanced regenerative surgery facilities.

RegenMed operating theater

- Our review of the literature surrounding the use of regenerative technologies in the surgical field, establishes the current advancements, the future direction of this field and how the modern operating theater can incorporate technology to facilitate the implementation and development of regenerative surgery.
- The field of RegenMed, as applied to surgical disease, is a rapidly changing field and this would need to be reflected in the design of any future operating suite with RegenMed in mind.
- The theater needs to be highly adaptable to account for significant advances that we can expect within the field over the next few years; current operating theaters are poorly equipped to deal with the scientific advances in tissue engineering.

Conclusion

- Development of purpose built, combined operating suites and tissue-engineering laboratories will provide the facility for the modern surgeon to treat patients with tissue or organ deficits, using bespoke, regenerated constructs without the need for immunosuppression.

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