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Tissue engineering with the aid of inkjet printers

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Tissue engineering holds the promise to create revolutionary new therapies for tissue and organ regeneration. This emerging field is extremely broad and eclectic in its various approaches. However, all strategies being developed are based on the therapeutic delivery of one or more of the following types of tissue building-blocks: cells; extracellular matrices or scaffolds; and hormones or other signaling molecules. So far, most work has used essentially homogenous combinations of these components, with subsequent self-organization to impart some level of tissue functionality occurring during *in vitro* culture or after transplantation. Emerging 'bioprinting' methodologies are being investigated to create tissue engineered constructs initially with more defined spatial organization, motivated by the hypothesis that biomimetic patterns can achieve improved therapeutic outcomes. Bioprinting based on inkjet and related printing technologies can be used to fabricate persistent biomimetic patterns that can be used both to study the underlying biology of tissue regeneration and potentially be translated into effective clinical therapies. However, recapitulating nature at even the most primitive levels such that printed cells, extracellular matrices and hormones become integrated into hierarchical, spatially organized three-dimensional tissue structures with appropriate functionality remains a significant challenge.

Keywords: bioprinting, extracellular matrix, growth factors, regenerative medicine, scaffolds, stem cells, tissue regeneration

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1. Introduction

Tissue engineering, also referred to as regenerative medicine, represents the convergence of science, engineering and clinical disciplines in order to understand the underlying biology of tissue development, homeostasis and repair, and then apply this knowledge to develop therapies that re-establish tissue and organ function impaired by disease, trauma or congenital abnormalities. The ultimate strategy may be to use genetic engineering to controllably turn on primitive regenerative genes, exemplified in lower order vertebrates [1,2], but which are essentially inactive in adult humans [3]. Achieving this capability in a predictable and safe fashion, however, is unlikely to be realized in the foreseeable future. In the meantime, most other strategies under development are based on either: delivering directly into the body minimal sets of biological building blocks, including cells, hormones, extracellular matrix (ECM) and/or degradable scaffolds in various combinations as cues to induce and guide the body to repair itself; or, prior to transplantation, attempting to first culture combinations of these building blocks *ex vivo* into more organized neo-tissues/organs. Early approaches intermixed the building blocks in essentially homogenous distributions throughout such tissue engineered constructs. However, a popularly held belief has been that the capability to spatially control the component distributions would lead to significantly improved outcomes because spatially controlled patterns would be

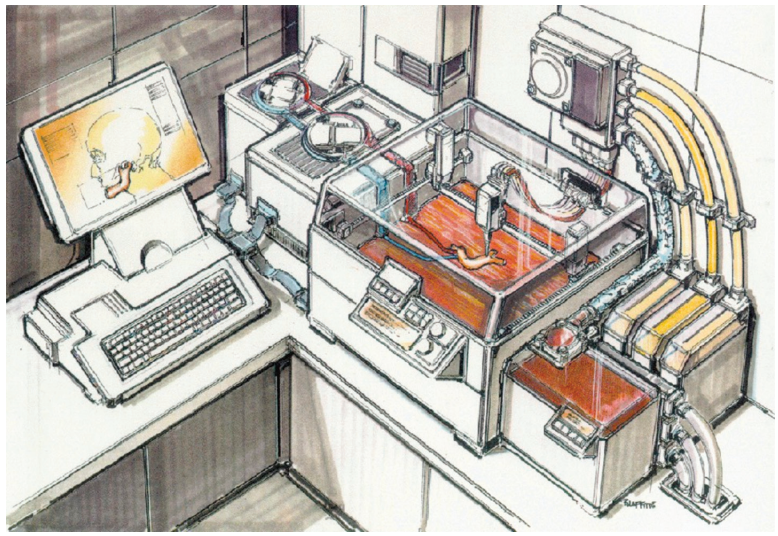

Figure 1. Conceptual vision of bioprinting system for manufacturing tissue-engineered constructs from 1995 [26].

more biomimetic (**Figure 1**). As a result, many groups, including the authors', began to develop computer-assisted 'bioprinting' technologies as a way to manufacture two-dimensional (2D) and 3D biological patterns. For this discussion, the authors define bioprinting as the selective deposition of 'bioinks' of biologically active components including proteins, peptides, DNA, cells, hormones (including cytokines, growth factors and synthetic hormonal signaling peptides), ECM molecules and native or synthetic biopolymers. Bioprinting holds great promise for tissue engineering, but these technologies are still in relatively early stages of development and have numerous hurdles to overcome to have real clinical impact.

2. Perspective

Bioprinting is an emerging field [4] representing diverse deposition processes, including, but not limited to, dip-pin writing, microstamping, photolithography, laser writing, electroprinting, microfluidics, electrospraying, stereolithography, microextrusion and inkjet deposition. Much of the bioprinting work has focused on 2D patterning for basic biological studies and is a logical antecedent to 3D printing. A critical step at these early stages of development is to demonstrate retention of biological activity of printed bioinks, retention of printed patterns over time and validation that the targeted biological activity is in register to printed patterns. As this basic groundwork is progressing, the extension to building 3D constructs has also been demonstrated for many of these approaches by incrementally building-up structures layer-by-layer, which is an idea borrowed from 'rapid-prototyping' methodologies [5].

Each bioprinting process being developed has advantages and disadvantages with respect to printing capabilities, including resolution, deposition speed, scalability, bioink compatibility and ease-of-use. The required specifications required for any given printed construct remains an open question. There is clearly no one best process and hybrid systems that combine the advantages of each are feasible. Although these technical capabilities are all important, the more important issue is that no one has yet to definitively demonstrate that bioprinting has lead to or will lead to therapies with improved clinical outcomes. The authors believe that bioprinted patterns will, at minimum, prove to have important applications as *in vitro* toolsets for basic biological discovery and cell screening assays, and that these capabilities will lead to improved therapy designs, even if they are only simple designs.

The research of the authors' group focuses on the use of inkjetting to print concentration-modulated patterns of growth factors on native ECM substrates such as fibrin. The authors emphasize the importance of conducting in-depth studies to fully characterize printed patterns, including retained growth factor concentrations and bioactivities [5-7]. Fibrin is used not only because it is a provisional matrix for wound healing, but also because fibrin naturally binds and immobilizes many growth factors of interest. The authors' rationale for engineering such 'solid-phase' (or immobilized) patterns is based on nature. Endogenous solid-phase extracellular growth factor patterns, including gradients, have been reported in developmental models [8-10]. Solid-phase growth factors are enabled because many growth factors exhibit inherent binding properties to ECM molecules, directly or through specific binding protein intermediaries [11]. Growth factor sequestration in the ECM can mediate spatial control by sequestering growth factors at specific locations within the ECM to create persistent patterns [9,12-14]. Other groups have also reported on inkjet-based bioprinting for patterning a wide variety of bioinks, including ECM molecules and antibodies [15], ECM and cells [16-18], enzymes [19], growth factors [20] and DNA [21].

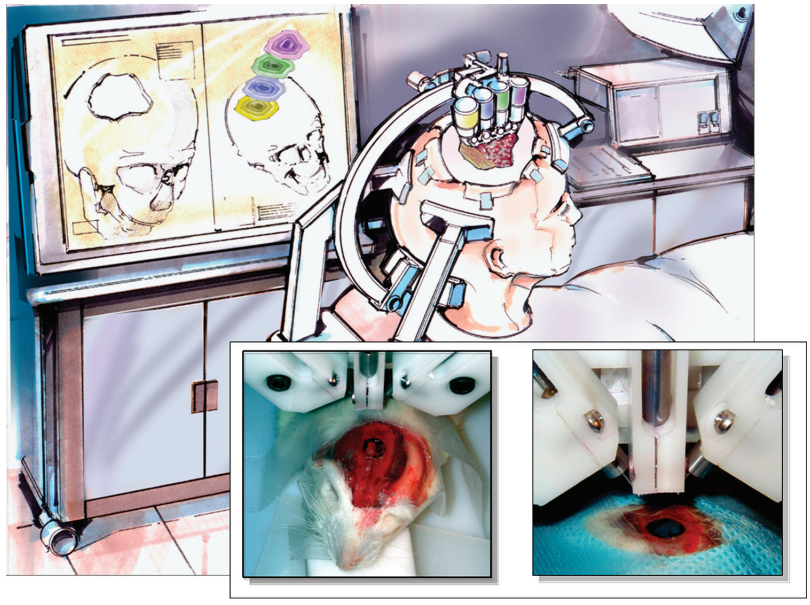

Figure 2. Conceptual vision of *in situ* bioprinting, with feasibility demonstrated by inkjet printing fibrinogen, thrombin and visualization dye into a rat calvarial defect (insert).

The authors selected inkjet deposition for several reasons. First, deposited concentrations of hormones can be easily modulated by overprinting individual locations with dilute bioinks [6]. Second, inketting is completely programmable, so custom templates are not required to create specific patterns, and therefore experimental turn-around times are relatively rapid. Third, an almost endless variety of bioinks can be deposited with inkjets. Fourth, inkjetting is non-contact, so there is less chance of contaminating substrates and printing on non-flat surfaces is also feasible. And, fifth, inkjetting is readily scalable. A possible disadvantage of using inkjetting is that it has lower resolution than photolithographic or microfluidic techniques, however, the authors have shown that the resolution achievable with inkjet printing is sufficient to produce cellular responses in register to printed patterns.

In contrast to simple spot patterns used in array technologies for proteomics and genomics [22], 2D inject printing of growth factors includes more complex shapes and pattern combinations for broader applications intended to direct behaviors of cell populations. In particular, directing the fate of stem cell populations is fundamental to the success of any regenerative application. In this respect, the authors have demonstrated that printed growth factor patterns on biologically relevant ECM substrates and direct cell fate in register to patterns, including cell proliferation, migration, apoptosis and differentiation [6,7,23,24]. Such experimental approaches represent potentially efficient methods for: screening growth factors; determining dosages and combinations for subsequent *in vivo* investigations and therapy development; and discovery for stem cell culture conditions for both expansion and differentiation. For example, using a simple printed pattern of bone morphogenetic protein-2, the authors demonstrated the potential to controllably engineer an uncommitted stem cell population toward two different tissues types, muscle off-pattern and bone on-pattern, by creating distinct but abutting microenvironmental niches [24]. In another possible application, relatively simple biological patterns, such as gradients, are well recognized in biology for directing development and when incorporated into a tissue engineered constructs, gradients may be useful patterns to direct endogenous stems cells into wound sites. Whereas replicating controlled persistent gradient patterns with soluble growth factors is problematic, especially *in vivo* and at the length scale of millimeters to centimeters, printed persistent growth factor gradients are straightforward to create with inkjet printing [6].

At present, although the authors' focus is on cell response to 2D patterns, solid-phase patterning methodology is extensible to 3D constructs. In the authors' approach, both the ECM and the growth factors are co-jetted; however, because gelled fibrin cannot be jetted the authors use multiple print heads for independent and concurrent deposition of fibrinogen, thrombin and growth factor bioinks. 3D fibrin/hormone structures are built-up, layer-by-layer, by jetted droplets mixing and gelling locally at the printed surface [5]. 3D patterns may provide better models for cell studies because they are more representative of cellular microenvironmental niches. Printing hydrogel-based constructs for extended *ex vivo* culture is clearly feasible. However, printing these constructs for immediate therapeutic delivery remains challenging, in part due to storage issues and poor surgical handability properties. These limitations could be technically overcome by inkjettng directly into the body using *in situ* printing. Although the

authors have demonstrated feasibility of *in situ* bioprinting (**Figure 2**), the authors do not believe that this would be a practical approach for many reasons, not the least of which is that clinicians want simple off-the-shelf solutions. Therefore, the authors are exploring new ways to shape, pattern or print onto constructs based on plastic forms of fibrin, with material properties ranging from elastic to hard. These plastics are synthesized using molding technologies originally developed during World War 2 [25].

Accurately forecasting the state of inkjet bioprinting technologies over the next 20 years and longer is difficult given the complexity of biological systems. The authors are confident that the technology capabilities will continually improve with respect to robustness, printing resolutions and achievable construct complexities. The authors can expect to see: more extensive use of 2D and 3D inkjetted patterns in various *in vitro* cellular assays in the next 5 years; extensive testing of bioprinted tissue constructs in animal models within the next 10 years; and testing in clinical trials within 15 – 20 years. In general, inkjet bioprinters will become more widely available to a broad range of investigators over the next several years. Therefore, it is likely that new unexpected applications will emerge as more investigators gain access to this technology.

3. Expert opinion

Returning to the vision of bioprinting in **Figure 1**, the question remains whether creating biomimetic tissue engineered constructs that recapitulate nature, even to a limited degree, will lead to significantly improved therapies, regardless if these constructs are immediately implanted or transplanted after culture? To be successful, significant challenges will have to be overcome. A fundamental problem for designing bioprinted constructs is that we have only a very limited understanding of the underlying biology of regeneration. Even as a more complete understanding is gained, it will probably be impractical to attempt to replicate all of the hundreds to thousands of factors involved in tissue repair. However, as tissue engineers gain new knowledge, this will provide them with the insight and intuition to help them select the minimum number of variables needed to create the simplest tissue engineered constructs capable of achieving desired clinical outcomes.

Another issue is that controlling the placement of molecules or cells within a construct will not insure that they will subsequently self-assemble into a functional tissue. Providing additional environmental cues will be required, including appropriate mechanical stresses, oxygen tensions, nutrients and other factors. Continued development of more sophisticated bioreactors will be critical for these applications. In addition, with the advent of large-scale engineered constructs will come the added complications associated with transplantation. In particular, large constructs will have to be anastimosed to the vasculature of the host to provide nutrients and remove waste if the transplanted construct is to survive and flourish. Laboratory-grown tissues will need mature vasculature branching topologies that lead out to large tissue engineered arteries and veins that will be easy and reliable to anastimose. Perhaps, advanced bioreactors will require artificial perfusion systems to support the *ex vivo* development of such vasculature. Foreseeably, nerves and lymphatics will eventually be included and their anastimoses will also have to be addressed.

Even if inkjet bioprinted neo-tissue constructs are realized experimentally, translation of these technologies into the clinic must overcome significant hurdles for FDA approval, be competitive in the market, gain clinician acceptance and satisfy demanding cost constraints associated with reimbursement and profitability. As the number of component types included in a construct increase, the timelines required for FDA approval will also increase, as will the costs to manufacture and market these products. Most importantly, inkjet bioprinted constructs will have to show clear cost advantages and improved therapeutic outcomes over existing 'off-the-shelf' solutions, such as allografts or synthetics, or simple constructs such as scaffolds delivering a single, uniformly dispersed growth factor.

For all of the aforementioned reasons it is unlikely that the vision of inkjet bioprinting depicted in **Figure 1** will become a clinical reality in the foreseeable future. Not realizing this or similar visions will not minimize the use of inkjet deposition and other forms of bioprinting. Bioprinting technologies offer unique strategies to controllably recreate microenvironments for improved 2D and 3D *in vitro* assays and modeling, especially in the context of stem cell physiology and the creation of simple neo-tissue constructs. Such *in vitro* applications hold clear potential to impact the development of more conventional, non-bioprinted tissue engineered constructs, as well as leading the way towards simple bioprinted constructs that may provide improved clinical outcomes. Finally, typical of any new platform technology, as yet, unforeseen benefits and new applications will emerge as inkjet and other bioprinting technologies become more broadly disseminated.

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