Inkjet Printing Technology for Regenerative Medicine

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Abstract

Tissue engineering and regenerative medicine has emerged as an innovative scientific field that focuses on development of new approaches for repairing cells, tissues and organs. While the current paradigm of utilizing combinations of biomaterial scaffolds and cells for tissue construction has shown to be effective, only a limited number of these technologies have been successfully translated to patients. This is due to various issues that are encountered in the tissue building process. Recent development of novel biomaterials, the discovery of new sources of cells, and advances in scaffold fabrication strategies are being applied to address these challenges. One of the challenges that hinder clinical translation is the inefficiency of current cell delivery methods. Living tissues maintain inherent multi-cellular heterogeneous structures, and rebuilding of such complex structures requires subtle arrangements of different cell types and extracellular matrix components at specific anatomical target sites. Inkjet printing technology has been proposed as a tool to address cell delivery concerns. In this session, this versatile method of building complex tissue structures will be discussed.

Introduction

Advances in tissue engineering and regenerative medicine have offered new therapeutic opportunities in the field of medicine in which conventional treatment modalities may be inadequate. Development of innovative technologies in tissue engineering has facilitated the maturation of translational therapies which allowed for the creation of cell and tissue substitutes that restore normal function. Although most of the developmental efforts remain in the experimental stages, some has been successfully translated to patients, which include the bladder, urethra and cell therapy for urinary incontinence.[1-4] The basic components used to achieve functional tissues and organs are cells, scaffolds and *in vivo* environment. Tissue engineering strategies that have been demonstrated to be successful involve the use of biocompatible matrices either with or without cells. The matrices are either used as supporting scaffolds to promote and enhance tissue regeneration over smaller distances, or as cell delivery vehicle for larger distances. When cells are used, donor tissue is dissociated into individual cells, which are expanded in culture, attached to a support matrix, and introduced into the body to form functional tissues. Cells for tissue reconstitution can be derived from the native organ to be replaced, thus avoiding rejection. In instances where normal tissues are not available, different cell sources may be explored. Stem cells offer numerous opportunities in the field of tissue engineering. Stem cells derived from various stages of development can be either implanted directly in the target tissues or guided into specific cell lineages in vitro followed by implantation in vivo.

Although individual tissue systems have been successfully engineered for various applications using the basic tissue engineering approach, building of complex tissues that consist of multiple cell and tissue components have not been established. This is due to various challenges encountered in the tissue building process. One of the challenges has been the inability to recreate the well-defined cellular configurations and functions of a native tissue. Living tissues contain multiple cell types and various extracellular materials arranged in specific patterns that are difficult to replicate *in vitro*. Thus, one important goal of tissue engineering and regenerative medicine is to develop a tissue fabrication method that allows specific control over the placement of various cells and matrices in three dimensions to mimic the complexity of native tissue architecture.

Emerging 'bioprinting' methodologies are being investigated to create tissue engineered constructs that initially have more defined spatial organization. The underlying hypothesis is that these biomimetic patterns can achieve improved therapeutic outcomes.[5] Bioprinting based on inkjet and related printing technologies can be used to fabricate these patterns and to study the underlying biology of tissue regeneration, leading to effective clinical therapies. Here, we present an overview of our recent work demonstrating the utility of inkjet technology in tissue engineering and regenerative medicine, which include the construction of multi-cellular functional tissues with adequate vasculatures, non-invasive monitoring of printed tissues *in vivo,* and gene delivery method for cellular function enhancement.

Inkjet printing of multi-cellular constructs

Although it has been reported previously that viable single cells can be “printed” using inkjet technology [2-4], simultaneous printing of multiple cell types for constructing heterogeneous tissue structures has not been demonstrated. To determine whether different cell types could be delivered to their designated target sites simultaneously, 3 different cell types were printed onto one platform to create a multi-cellular construct. The cells [canine bladder smooth muscle cells (SMCs), mouse aortal endothelial cells (ECs), and human amniotic fluid derived stem cells (AFSCs)] were marked with membrane bound fluorescent dye, loaded into separate cartridges and printed onto separate target sites in a “pie” shaped configuration. The printed 3D construct, containing the 3 cell types, was confirmed after printing. Greater than 90% of the printed cells survived during the bio-printing process. The three dimensional pie-shaped construct was then implanted into the subcutaneous space of athymic mice to determine the survivability *in vivo*. The tissue structure of the 3D configuration was maintained for 2 weeks after implantation. The membrane-bound tracers on individual cells confirmed that the printed cells remained viable in their pre-determined locations *in vivo*. These results show that multiple cellular components can be delivered to specific target sites using inkjet printing method.

Fabrication of functional tissues with adequate vasculature

Achieving cell and tissue function in vivo is the primary goal of regenerative medicine. To demonstrate that cell-printed constructs survive and form functional tissues with adequate vasculature *in vivo*, we have printed various cell types and implanted the resulting constructs. In one experiment, osteogenically differentiated stem cells derived from human amniotic fluid were printed and implanted in immune-deficient mice and followed for up to 18 weeks.[6] An unseeded control scaffold also was implanted in each mouse. After 8 weeks constructs were recovered and analyzed histologically using von Kossa's stain. Highly mineralized tissue was observed from the implanted cell-seeded scaffolds but not from the implanted unseeded scaffolds. After 18 weeks the generation of hard tissue within the printed constructs was evident. Blocks of bony tissue were observed at the site of implantation with density somewhat greater than that of mouse femoral bone. This experiment demonstrates that inkjet printing can be used to engineer functional tissues in vivo.

Vascularization is a critical component required for tissue survival *in vivo*. In another experiment, endothelial cells (EC) and smooth muscle cells (SMC) were used as a printing medium in order to achieve adequate vascularization of muscle tissue *in vivo* as EC are known to promote angiogenesis. Subsequent implantation of printed tissue constructs resulted in formation of a vascular network within the engineered tissue, which was confirmed by visual and microscopic examination. These vessels expressed the endothelial cell-specific marker von Willebrand factor (vWF). In addition, the printed SMCs matured into elongated muscle fibers that were aligned in unidirectional orientation 2 weeks after implantation. Immunohistochemical analysis of the muscle fibers indicated consistent expression of α-smooth muscle actin. These results indicate that addition of angiogenic factors, such as endothelial cells is able to enhance vascularization of the printed engineered tissue in vivo.

Non-invasive monitoring of the development of printed tissues *in vivo*

An important task following tissue fabrication is to monitor tissue development and maturation in recipients. Presently, methods to monitor this process have not been adequately established. This may be due to a lack of adequate assessment tools that permit close monitoring of tissue function and integration into host tissues over time. In addition, most of the current methods of tissue evaluation rely on examining retrieved implants at various time points, which requires the sacrifice of the host. Thus, this would not be practical in the clinical setting.

Magnetic resonance (MR) imaging is a noninvasive tool that provides anatomical and functional images with exceptionally high spatial resolution. We have explored the possibility of developing a suitable MR imaging technique for the assessment of printed tissue implants.[7] Layer-by-layer inkjet printing technology was used to fabricate three different tissue constructs on alginate/collagen gels: bovine aortic endothelial cell (EC)-printed (to represent soft tissue), human amniotic fluid derived stem cell (AFSC)-printed (to represent hard tissues as they underwent osteogenic differentiation *in vivo*), and cell-free constructs (scaffold only). The constructs were subcutaneously implanted into athymic mice and regularly monitored using a 7T MRI scanner.

The three tissue construct types showed distinct image contrast characteristics due to the different tissue microstructures and biochemical compositions present at various time points. The soft tissue constructs (EC-printed) showed the highest signal intensities in the T2-weighted images. One possible explanation lies in the differences in water content, and therefore proton content, in the different types of tissue. Because living cells are mainly composed of water (60-80% in volume), the number of cells that present in a construct could easily change this parameter. In contrast, the hard tissue constructs (AFSC-printed implants) showed the darkest appearance in the T2-weighed images and contrast. This may have resulted from the osteogenic differentiation and mineralization of the AFSC-printed constructs *in vivo*. This mineralization process is dominant and greatly restricts the movement of water molecules into the implant, which differs from the soft-tissue implant where fluid can move freely. Therefore, the mineralized AFSC-printed constructs exhibited relatively low T2 signal intensity on MR images, leading to a "dark" image. It was also shown that, at different time points, the same tissue constructs exhibited different MR characteristics. The EC-printed implants had significantly higher T2 values at the 10 week versus the 5 week post-implantation. This may be associated with the levels of collagen content.

Tissue vascularization is critical for the viablity of engineered tissues after implanation. Conventional analytical methods, such as histology and immunohistochemistry, can provided limited information on a single static state of tissue vascularization, but dynamic or funtional analysis is difficult to obtain through these invasive methods. Non-invasive methods are required to evalute the functionality of vascularization in the cell-printed constructs if these constructs are to be used for larger-scale pre-clinical and clinical trials. To evaluate the dynamics and functionality of the observed tissue vascularization, dynamic MRI was carried out with intravenous (IV) injection of Gd-DTPA, a gadolinium-based contrast agent visible in MRI images. In this study, the vasculature was clearly observed in the EC-printed implants using MR imaging with contrast perfusion. Interestingly, dynamic or functional characteristics can also be measured using this method. Pre- and post-contrast T2-weighted images showed enhanced visualization of the vasculature within the implants after the intravenous injection of contrast media. These data suggest that the EC-printed implants formed functional vasculature *in vivo*, and that this vascular network was able to integrate into the host's vascular system. All of these results indicate that high resolution MR imaging is a promising method for noninvasive, long-term monitoring of the status of cell-printed construct growth, differentiation, and vascularization.

Inkjet mediated gene transfer into living cells combined with targeted delivery

Currently, the main goal of regenerative medicine therapies is to restore normal tissue function. In instances where normal tissue cannot be engineered with available cell types or enhanced cellular function is required to achieve a desired therapeutic outcome, alternative approaches such as growth factor supplementation, macromolecule treatment, or gene modification may be required. Moreover, in tissue engineering the transfection or delivery of functional genes into target cells to facilitate the formation of functional tissues and organs is becoming vital.

In various areas of research, gene modification techniques have been used to improve cell and tissue function. Although there are established methods for delivering genes into cells, such as viral transfection, microinjection, electroporation, and liposome mediated transfection, the application of these techniques to tissue engineering has been difficult due to either significant viral toxicity (e.g. transfection using viral vectors), loss of cell viability (e.g. electroporation), or low transfection efficiency (e.g. microinjection and liposome mediated method). In addition, the existing techniques require pre-processing of the cells prior to building new tissues for therapy. Therefore, there is a need to develop methods to effectively and efficiently transfect cells with specific genes during the tissue building process without compromising cell viability. This could be achieved by combining transfection and cell delivery on one tissue engineering platform.

Droplet formation and ejection in thermal inkjet printing entails elements of high heat (up to 300° C) and velocity-induced sheer stress (up to 10 ms-1). Due to the short time required for nozzle firing (< 20 μs), cells survive passage through the printer nozzles and remain physiologically and functionally normal. However, during printing, transient distortion of the cell membrane with associated alteration of permeability may occur. It is well-established that brief application of electric fields or hydrodynamic pressure leads to changes in cell membrane permeability that allow the introduction of DNA and other macromolecules into cells. With this in mind, we hypothesized that inkjet printing of cells could cause a similar temporary disruption of the cell membranes and facilitate intracellular delivery of plasmid vectors.

We have performed simultaneous transfection with a plasmid vector and delivery of cells into two and three dimensional tissue constructs using an inkjet printer.[8] Plasmids encoding green fluorescent protein (GFP) were co-printed with living cells (porcine aortic endothelial (PAE) cells) through the ink cartridge nozzles of modified commercial inkjet printers. The PAE cells co-printed with plasmid DNA retained their characteristic morphology and a subpopulation of the cells showed strong green cytoplasmic fluorescence, demonstrating successful transfection with the pmaxGFP plasmid. The transfection efficiency of the printed cells, determined by GFP expression, was over 10% and post-transfection cell viability was over 90%. We showed that printing conditions, such as plasmid concentration, cartridge model, and plasmid size influenced gene transfection efficiency. Moreover, genetically modified PAE cells were accurately delivered to target sites within a three dimensional fibrin gel scaffold and expressed GFP *in vitro* as well as *in vivo* when implanted into mice. These results demonstrate that inkjet printing technology is able to simultaneously transfect genes into cells as well as precisely deliver these cell populations to target sites. This technology may facilitate the development of effective cell-based therapies by combining gene therapy with living cells that can be delivered to target sites.

Conclusion

In this review we present various utilities of inkjet printing technology in the tissue building process. We show that viable three-dimensional heterogeneous tissue constructs made from multiple cell types can be created by printing cells and hydrogel scaffold materials layer-by-layer. The cell-printed constructs are able to survive and mature into tissues with adequate vascularization when implanted *in vivo*. Moreover, *in vivo* development of cell-printed constructs with tissue vascularization can be monitored by high magnetic field MRI. In addition, genes can be transferred by co-printing living cells with plasmids while delivering them to target sites accurately. These novel methods used for creation of multi-cellular tissues with enhanced function may address some of the current challenges facing the translation of regenerative medicine therapies. However, the use of inkjet printing technology requires extensive testing and validation in order to be applied clinically. These include improvements in the existing printing systems, optimization of biomaterial scaffolds, and incorporation of functional molecules into "bioink" to improve cellular functionality.

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Tao Xu received his BS in polymeric materials from Beijing University of Aeronautics & Astronautics, China (1994) and his PhD in bioengineering from Clemson University, SC, USA (2005). Since then he has worked as a postdoctoral fellow at the Wake Forest Institute for Regenerative Medicine, NC. His current research interests include cell and organ printing for regenerative medicine.