Directed assembly of cell-laden hydrogels for engineering functional tissues

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Introduction

Regenerative medicine is an emerging branch of medicine that aims to cure patients suffering from severe tissue and organ failure. Regenerative medicine has the potential to resolve the problems associated with transplantation such as the shortage of donor organs and the immune rejection. Despite significant progress in this area, however, three key challenges remain in generating artificial tissues for clinical use.1 These include: (1) the inability to obtain an adequate number of organ-specific cells, (2) the inability to generate functional tissue constructs and (3) the lack of vasculature in engineered tissues.

With the recent advances in stem cell biology, it is becoming more realistic to obtain an adequate number of autologous or immunologically compatible cells. However, only a small fraction of injected cells are integrated into the host organs. To address this issue, engineering tissues using these cells before transplantation may be an important strategy. There have been many examples that utilize biodegradable porous scaffolds to engineer tissues, a few of which have already been used in the clinic.2 While this approach has been widely used and has shown to be useful,3 it requires cells to rearrange themselves for generating tissue-like complexity. This re-organization process, which is often essential for recreating the desired tissue function, would benefit from engineering systems that aim to aid it in recreating biomimetic tissue complexity. An emerging approach to generate tissues with tissue-like microarchitectures is based on the directed assembly of functional tissue units. This process aims to assemble cell aggregates or cell-laden structures for creating biomimetic hierarchical structures.4

In this paper, we discuss the latest progress in directing the assembly of cell-containing structures to generate engineered tissues. We will first discuss organ structure and scaffold-based tissue engineering, followed by fabrication approaches of cell-laden microgels as building blocks. We will then discuss different methods for directed assembly of cell-laden hydrogels. The paper will be concluded with the future perspectives to directed assembly technology.

Organ structure and tissue regeneration. Billions of cells are arranged in a highly organized manner to function as tissues.5,9 During tissue development, formation of organ-specific tissue microarchitecture is influenced by spatio-temporal micro-environmental factors such as physical forces and chemical cues. Reciprocally, the tissue architecture guides the tissue development and function. The role of tissue microarchitecture is highly related to its resulting function and thus any attempts at recreating engineered tissues must aim to recreate biomimetic tissue structures.

Most tissues generate an organized complex architecture by assembly of repeating functional tissue units over several scales. Examples of functional tissue units are islets in the pancreas, nephrons in the kidney and lobules in the liver.10 These functional tissue units are highly vascularized and contain many different cell types that are positioned relative to each other in a highly organized manner. Given this complexity, the architecture

Tissue engineering aims to develop functionalized tissues for organ replacement or restoration. Biodegradable scaffolds have been used in tissue engineering to support cell growth and maintain mechanical and biological properties of tissue constructs. Ideally cells on these scaffolds adhere, proliferate and deposit matrix at a rate that is consistent with scaffold degradation. However, the cellular rearrangement within these scaffolds often does not recapitulate the architecture of the native tissues. Directed assembly of tissue-like structures is an attractive alternative to scaffold-based approach for tissue engineering which potentially can build tissue constructs with biomimetic architecture and function. In directed assembly, shape-controlled microstructures are fabricated in which organized structures of different cell types can be used as tissue building blocks. To fabricate tissue building blocks, hydrogels are commonly used as biomaterials for cell encapsulation to mimic the matrix in vivo. The hydrogel-based tissue building blocks can be arranged in pre-defined architectures by various directed tissue assembly techniques. In this paper, recent advances in directed assembly-based tissue engineering are summarized as an emerging alternative to meet challenges associated with scaffold-based tissue engineering and future directions are addressed.

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of the initial cell-laden construct is important for reacting this tissue structure.

Scaffold-based tissue engineering largely relies on migration, proliferation and differentiation of cells in a biodegradable porous scaffold. Although cells have a great capacity to rearrange and reorganize upon seeding on tissue engineering scaffolds, they may not fully reorganize themselves into tissue-like functional units. Directed assembly-based tissue engineering could be a potentially powerful tool to overcome these challenges and to recreate tissue complexity. In this approach, microfabrication techniques are used to control and organize the relative position of individual cells in length scales of around 1–10 μm while these fabricated constructs are assembled as building blocks that are in the order of 10–1,000 μm to obtain biomimetic repeating units. This assembly is further coupled with approaches such as micromolding of hydrogel microchannels to fabricate vascular structures in order to engineer milli- and centimeter-scale tissue constructs. Thus the directed assembly-based tissue engineering has significant potential to generate tissue constructs with controlled structures that range from microscale to organ level.

Scaffold-based tissue engineering. Biodegradable scaffolds have been commonly used for engineering tissue constructs. A scaffold is a structural support that facilitates the formation of tissues from seeded cells.\(^{30-36}\) The scaffold ideally degrades at a rate similar to the rate of cell growth and matrix deposition such that it will completely be replaced by engineered tissue. Various scaffold features, such as structure, physical cues and mechanical properties can be modulated to regulate the tissue development and remodeling.\(^{31,37}\)

Although scaffold-based tissue engineering is well established and proven to be useful, it has faced some challenges to build tissue constructs with biomimetic architecture and function. Cell-ECM interactions and cell-cell signals from specialized cells within close proximity play significant role to shape the microenvironment, to preserve the cell phenotype and to direct the cell differentiation. Therefore, the arrangement of cells as well as the structure and composition of microenvironment are important in maintaining the tissue structure and function. However, the cellular rearrangement within scaffolds often does not resemble the biomimetic structure of the native tissue. In addition, cell seeding of the scaffold as well as subsequent vascularization are major hurdles. These become even more complicated considering that organs contain multiple cell types in close proximity which are organized within microscale resolution in multiple scales.

Directed assembly-based tissue engineering. Directed assembly-based tissue engineering aims to address the uncontrolled arrangement of cells and remodeling of microenvironment structure. This approach also has the potential to address the tissue vascularization. Directed assembly employs biomimetic techniques by fabrication of small microstructures to arrange different cell types in close proximity of each other. These building blocks can be made from cell aggregates or cell containing hydrogel blocks.\(^{38-23}\) Hydrogel encapsulated cells can mimic functional tissue units so that the tissue function may be potentially preserved by the arrangement of these building blocks in a desired geometry with specific shape and size. Directed tissue assembly is a promising approach which provides enhanced level of control to organize tissue building blocks into desired patterns which may enhance the functionality of the final tissue construct. There are different techniques for making microengineered cell-laden hydrogels as tissue building blocks, which are discussed in the following section. It is important to note that cell aggregates are also a suitable building block of tissue-like constructs which can be directly assembled to form a tissue construct. Although these techniques are not discussed here, the readers are directed to a number of recent works in generating cell aggregates with controlled shape and size.\(^{19,20}\)

### Fabrication Techniques for Engineering Microscale Hydrogels

Cell-cell and cell-ECM (extracellular matrix) interactions shape the biomimetic structure of microenvironment which can direct the tissue structure and function. To provide a three-dimensional (3D) environment for cells, biocompatible hydrogels have been extensively used for engineering tissue constructs.\(^{21,22}\) Hydrogels are 3D crosslinked networks of hydrophilic polymers with high water content. In general, hydrogels are highly permeable to oxygen, nutrients and other water-soluble metabolites\(^{23}\) which make them ideally suited for cell encapsulation since they mimic the natural ECM.\(^{24}\)

Hydrogels can be derived from either natural sources such as fibrin,\(^{25}\) hyaluronic acid (HA),\(^{26}\) and agarose;\(^{27}\) or be synthetically formed such as poly(ethylene glycol) (PEG).\(^{28,29}\) Both natural and synthetic hydrogels have been used for cell encapsulation and have been engineered to create structures with features between 0.1–10 mm.\(^{30}\) A number of different techniques have been developed for controlling the shape of hydrogels with microscale resolution (i.e., microgels). These approaches utilize technologies such as emulsification, photolithography, microfluidics, micromolding, modular assembly and printing. Here we describe each of these techniques in detail.

**Emulsification** is a widely used technique for generating microgels. In this process a multi-phase mixture is stirred to generate small aqueous droplets of the hydrogel precursors within an organic phase.\(^{30}\) Aqueous droplets are subsequently gelled by crosslinking via various mechanisms to generate spherical microgels (Fig. 1A). This approach is highly scalable and can be used to easily generate microgels. However a key limitation of emulsification is that it is often limited to spherical microgels while a relatively wide distribution of microgel sizes is usually generated.

**Photolithography** is a technique that has been widely used in the microelectronics industry. In this process a thin film of a polymer is exposed to light through a mask. As the light reaches the photosensitive polymer through the transparent regions of a mask, polymer will be crosslinked through photopolymerization\(^{30}\) (Fig. 1B). Synthetic and natural photocrosslinkable pre-polymers have been developed to make hydrogels while a number of studies have demonstrated that photocrosslinkable microgels made from various types of synthetic polymers such as PEG\(^{31,32}\) can be used for cell encapsulation.\(^{33}\)
Microfluidics systems can also be used for generating microengineered hydrogels. Microfluidic devices which operate based on the movement of fluids through microscale channels provide a unique platform for generating hydrogels with controlled features. A common method of using microfluidic systems to generate microparticles is through the use of multiphase system. In this approach, the surface tension and viscosity are used to create homogeneous particles that can be cross-linked to form microscale hydrogels. Interestingly, microfluidic techniques and photolithography have been merged to generate other methods of fabricating microscale hydrogels by using the channels to deliver the hydrogel precursors to a region where they gel by light exposure. This approach can be used to generate microgels with capability of controlling their shapes and sizes in a continuous manner.

Micromolding is another technique for generating hydrogels with controlled features. Micromolding has become particularly appealing due to the development of methods such as soft lithography that has enabled easy fabrication of poly(dimethyl siloxane) (PDMS) molds from prefabricated silicon wafers. To generate micromolded hydrogels, precursor polymers are initially molded and subsequently gelled to generate structures with different shapes and sizes. Micromolding has also been used to generate microengineered hydrogels from a variety of materials including hyaluronic acid (HA), chitosan and PEG. In addition, micromolding techniques have been recently adapted to micromold hydrogels such as alginate and fibrin that require the addition of crosslinkers such as divalent cations. These materials, which encompass a large class of hydrogels, were molded by using hydrogel micromolds that were capable of simultaneously controlling the shape of the hydrogel precursors and the delivery of crosslinking agents.

Printing technology can also be used to encapsulate cells in hydrogels. In this process, standard or modified printers are used where paper and ink are replaced with substrate (usually polymer made) and cell-laden medium respectively. In all of the hydrogel-based building block fabrication techniques that have been discussed so far in this section, the building blocks are first fabricated and then they will be organized in specific geometry using directed assembly. In contrast, in printing method, both steps of fabrication and assembly of hydrogels are merged. We discuss the tissue printing technique in detail in the next section. In Table 1 we summarize some of the applications as well as pros and cons of these different methods.

Directed Assembly of Cell-Laden Hydrogels for Engineering Tissue Constructs

Directed assembly can be used to arrange tissue building blocks in a biomimetic manner to aid in subsequent tissue formation through dynamic tissue remodeling. The microengineered building blocks are expected to maintain both biomimetic architecture and function of the encapsulated cells which can be achieved by controlling cell-microenvironment interactions through substrate mechanics, biomaterial chemistry, applied physical forces and the degradation of surrounding materials.

Different techniques for fabricating hydrogel-based tissue building blocks were discussed in the previous section. In this section, we discuss different directed assembly techniques to assemble hydrogel-based building blocks into a tissue construct with controlled geometry and functionality.
Directed assembly at a two-phase interface. Our group has developed a number of approaches for using directed assembly to build tissue-like constructs with well-defined macroscale architectures from cell-laden microgels. For example, the directed assembly of cell-laden microgels can be achieved by harnessing the surface tension characteristics of hydrophilic hydrogels in a two-phase oil-aqueous solution reactor.\textsuperscript{43} (Fig. 2A–F). First, cell-laden hydrogels were created in various sizes and shapes through photolithography. These micro-tissue modules were subsequently transferred to a hydrophobic mineral oil and mechanical agitation was applied to induce the aggregation of the hydrophilic microgels to form tissues of varying dimensions. The microgel assembly could then be stabilized through a secondary photo-crosslinking process.\textsuperscript{52,53} Directed assembly of cell-laden microgels within aqueous medium is of great benefit for building tissue constructs with clinical significance. Du et al. achieved directed assembly of cell-laden microgels within aqueous droplets on surfaces patterned with hydrophobic and hydrophilic regions.\textsuperscript{45} Driven by surface-tension, the hydrophilic microgels assembled within patterned aqueous droplets, which are confined inside the hydrophilic patterns and stabilized by a secondary crosslinking (Fig. 3A). Using this process microgel assemblies with well-regulated architecture were formed on the glass slide by controlling the size and shape of the surface patterns (Fig. 3B and C). The stabilized assembled microgels could be readily harvested from the surface as multi-cellular tissue constructs (Fig. 3D). This “bottom-up” approach enables rapid and scalable fabrication of cell-laden microgel assemblies with predefined geometrical and biological features, which can potentially be used for microscale tissue engineering applications. Restricted in the flattened surface patterns, the final structure of the microgel assembly produced by this approach is limited to

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<th>Techniques</th>
<th>Application</th>
<th>Advantage</th>
<th>Disadvantage</th>
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<tr>
<td>Emulsification</td>
<td>-Immunosolulation.</td>
<td>-Easy to generate microgels.</td>
<td>-Larger size distribution in the gel than other synthesis approaches.</td>
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<td></td>
<td>-Carriers within bioreactors.</td>
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<td>-Little control of the resulting shapes (spherical droplets).</td>
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<td></td>
<td>-Analyzing stem cell biology.</td>
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<tr>
<td>Photolithography</td>
<td>-Making 2D structures.</td>
<td>-Easy and rapid to generate microgels.</td>
<td>-Effect of photocrosslinkable materials and UV irradiation on cell function.</td>
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<td>-Create microstructured hydrogel scaffolds.</td>
<td>-High resolutions (micron scale to millimeters).\textsuperscript{52,53}</td>
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<td>-Conjugate chemical entities to hydrogels with controlled spatial resolution.</td>
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<td>Microfluidics</td>
<td>-Generate Janus particles (i.e., particles with two or more distinct sides).</td>
<td>-Creating particles with variety of sizes and shapes.</td>
<td>-Requirement of sophisticated equipments.</td>
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<td>-Create hydrogels with controlled gradients of signaling molecules or material properties embedded in the hydrogel.\textsuperscript{38}</td>
<td>-Possible to merge with other methods like photolithography.\textsuperscript{56,67}</td>
<td>-Relatively low throughput.</td>
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<td>-Fabrication of hydrogels with engineered barcodes for biosensing and high-throughput applications.\textsuperscript{56}</td>
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<td>Micromolding</td>
<td>-Drug delivery.</td>
<td>-Generating 3D hydrogel microstructures.</td>
<td>-Hydrogels may swell upon release causing different sizes than features on the PDMS mold.\textsuperscript{68,70}</td>
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<td>-Nano-scale topography of hydrogels will be important in generating improved tissue engineering scaffolds.</td>
<td>-Simply generate a variety of micromolded structures.</td>
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Table 1. Comparison of different techniques for generating hydrogel-based building blocks.
two-dimensional (2D) structures, which is applicable to mimic simple tissues (i.e., skin) while it lacks the complexity of 3D tissue structures.

Recent progress has been made to expand the directed assembly approach to generate 3D structures with higher complexity. Fernandez et al. developed a directed assembly process which occurs on a hydrophilic surface. The PDMS surface was used as a hydrophilic template which attracts and confines the microgel subunits. As shown in Figure 3E, microgels made with specific shapes were first mixed in a pre-polymer solution and deposited on an oxygen-plasma treated PDMS surface. Due to hydrophilic affinity, the liquid wets the surface and drives the microgel subunits to cover the PDMS surface. Upon removal of the excess pre-polymer solution, microgels assemble into a closely packed sheet.
induce the directed assembly of cell-laden microgels into defined geometries and shapes within physical barriers. Bruzewicz et al. directed the assembly of cell-laden microgels by loosely packing the modules into microfluidic chambers (channels) as a physical template. The microgel assemblies exhibited structures defined by the physical template (Fig. 4A–C). The order in the packed assemblies increased as the width of the microchannels approached the width of the modules. The physical chamber allowed perfusion of the tissue assembly through the interconnected pores. One benefit of this assembly approach is the ability to form an assembly that contains multiple types of cells. The ability to precisely organize different types of cell-laden microgels into a 3D array enables fabrication of 3D tissue constructs with hierarchical structures. Artificial tissues with different structures containing multiple cell types (i.e., HepG2 cells and 3T3 fibroblasts) can be spatially organized in the small microfluidic channels (Fig. 4D). Recovery and
An attractive technique to modularly assemble the large porous cell-laden hydrogels is introduced by Liu et al. Poly(ethylene glycol) diacrylate (PEGDA) made cell-laden microgels were first fabricated using photolithography, and then physically bonded in the presence of a cross-linker carrying multiple thiol groups through a Michael-type addition reaction. The tissue construct was assembled through the reaction between the thiol groups on the crosslinker and the acrylate groups on the surface of the building block microgels. It was reported that by using this approach, cells were uniformly distributed and remained analysis of modules after 24 h under constant flow of medium showed that over 99% of encapsulated cells survived this interval in the microfluidic chamber. The physical template-based assembly enables the control of the final geometry of the microgel assembly but it is hard to control the order of microgel modules within the assembly when the template is much larger than the cell-laden modules.

Physical manipulation of individual microgels into ordered patterns was achieved by using a micromanipulator. An example is an alternating checkerboard pattern (Fig. 4E), assembled with fluorescently red- and green-stained cells in separate sets of microgels. This approach is relatively inefficient and non-scalable for fabrication of large-scale tissue due to difficulties encountered in manipulating individual microgels.

McGuigan et al. assembled the modular components, consisting of submillimeter-sized collagen gel rods seeded with endothelial cells (ECs) in a chamber to build micro-vascularized tissue (Fig. 4F). The EC-covered collagen modular units (with HepG2 encapsulated) were assembled into a larger tube and perfused with medium or whole blood. The resultant tissue assembly consists of interconnected cellular modules, which are perfusable and non-coagulative due to endothelialization (Fig. 4G and H). Random assembly of microgel modules is rapid and simple, but lacks the control over the final structure of the hydrogel aggregate.

Microfluidic-directed assembly is a promising approach for parallel fabrication of devices made up of many small components with high degree of accuracy. Chung et al. developed “railed microfluidics,” a fully deterministic way to guide and assemble microstructures inside fluidic channels. They fabricated grooves (rails) on the top surface of the PDMS channels as guidance for the movement of microstructures which were fabricated with complementary polymeric microstructures to fit with the grooves (Fig. 5A).

By using the rails as a guiding mechanism, complex one- and two-dimensional microsystems, each composed of more than 50 hydrogel-based microstructures (each sized smaller than 50 μm), were fluidically self-assembled (Fig. 5B). Furthermore, they achieved heterogeneous directed-assembly of two types of living cell-laden hydrogel building blocks by taking advantages of rail-guided self-assembly and cross-solution movement (Fig. 5C and D). Owing to its simplicity and flexibility, railed microfluidics will not only impact current self-assembly but also encourage innovation in a wide range of application areas in the future.
and growth factors is provided. Clusters of several thousand of cells are encapsulated in microgels to form droplets which can be printed according to the desired 3D computer model. Cells in encapsulated microgels fuse, arrange themselves and self-assemble to form a tissue-like construct similar to the computer design when they are placed close to each other.

For sterile use bio-printer should fit inside a standard biosafety cabinet. Some of them have two print heads, one for placing cells and another for printing a hydrogel matrix. A typical bioprinter is equipped with a software interface to design the computer model of desired tissue and a laser calibration system, which may be controlled by computer to position the print head within micrometer precision.

The applications of inkjet technology to tissue printing have been rapidly advanced in past few years; however the usage of inkjet tissue printing technique is hampered by some limitations including the size of the jetting needle, the cell concentration, the viscosity of the material and the viability of the printed cells. Although inkjet tissue printing has faced some shortcomings, there are some other jet-based techniques which can overcome disadvantages of the inkjet printing.

Electrospray or electrohydrodynamic jetting (EHDJ) and electrospinning are emerging alternatives to inkjet printing. EHDJ is a physical phenomenon which is generated by applying a potential difference between a grounded electrode and a needle filled with a liquid medium. The medium exiting

Figure 5. Microfluidic-guided assembly of cell-laden hydrogel building blocks. (A) Guided movement of microtrains along a sinusoidal-shaped rail in a microfluidic channel. (B) The complex self-assembly such as Greek temple was completed simultaneously by applying fluidic force inside a railed microfluidic channel. Each microstructure (37 microstructures in total) is fabricated independently on a corresponding rail. (C) Heterogeneous assembly process on the basis of the cross-solution movement scheme. (D) Assembled 3 x 3 matrix with two different living cells using cell-containing PEG-DA hydrogel building blocks.
the needle goes through a high-intensity electric field where jet droplets will form. The size of jet droplets depends on the flow rate and properties of the liquid medium and the potential difference between electrode and the needle. EHDJ and Electrospinning have recently been successfully employed to print living cells by Jayasinghe et al. They could deposit cell droplets with a few microns diameter to make viable tissue constructs. Electrospaying and electrospinning could overcome the shortcomings of inkjet tissue printing such as size of droplets, resolution and cell viability. These techniques can be potentially employed for precise cell deposition with applications of tissue fabrication at the micrometer and nanometer scales.

**Conclusions and Future Perspectives**

Directed assembly of tissue constructs is an emerging strategy with appealing results for engineering functional tissues. This approach aims to mimic the structure of native tissues often constructed of multiple functional units organized in multiple scales with specific geometry to preserve tissue function. Encapsulated cells in hydrogels closely mimic tissue units which can be combined to make a large tissue construct with desired architecture and function through regulating the formation of local microenvironment while maintaining dynamic tissue remodeling. This can potentially be used to study the mechanisms of in vitro tissue organogenesis through designing tissue architectures capable of producing sufficient ECM and dynamic remodeling with predictable function.

In this method, populations of different cell types can be encapsulated in hydrogel blocks to form tissue units where these tissue units can be assembled to make a tissue construct with specific geometric patterns. Such technique preserves the desired shape and size of the microstructured building blocks while it provides a better control on spatial cell arrangement within these blocks.

Although microscale technologies enable precise control of the micro-architecture of the modular tissues, controlling the macro-architecture and cellular function of the tissue assembly remain as the major challenges of directed assembly-based tissue engineering. Other challenges to overcome in this field include: establishment of biomimetic microenvironments within the microgels (i.e., materials with biochemical and mechanical properties and functional microvasculature), scaling-up the assembly techniques for producing construct of tissue-level dimensions and successful integration and remodeling of the assembled tissues with the native tissue environment after transplantation. It is foreseen that directed tissue assembly possesses immense potential for advancing the field of tissue engineering and there will
be increased integration of directed assembly-based approach with conventional scaffold-based approach to create more complex functional tissues with physiological architecture for clinical applications. In future in vitro directed assembly of cell-laden hydrogels from autologous cells can potentially eliminate allogenic organ transplantation, patient waiting lists and complications after transplantation due to immunosuppressive therapy.

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