CHAPTER

7

# Self-Assembly of Cell-Laden Hydrogels on the Liquid-Air Interface

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## Abstract

The microarchitecture surrounding cells can have a substantial impact on cell function. Therefore in designing complex engineered tissues, improved control of the cellular microarchitecture can greatly improve the function of engineered tissues. The proposed procedure describes a bottom-up approach for fabrication of three-dimensional (3D) tissue constructs with the capability to better control the cellular microarchitecture. This platform can be used to fabricate 3D tissues through self-assembly of cell-laden hydrogels with tunable microarchitectural features. The fabrication process is controlled by the surface tension forces at the liquid-air interface. As a result, cell-laden hydrogels with a broad range of different sizes, shapes, and cell densities can be used to construct macroscale engineered tissues with specific microarchitectures in a rapid, high-throughput approach.

Key terms

bottom-up approach
controllable microenvironment
hydrogel
self-assembly
tissue engineering

## 7.1 Introduction

Tissue engineering is a promising way to address the limited supply of donor tissue. Many of the current approaches to engineer tissues are based on seeding cells on 3D scaffolds [1, 2]. However, some of the major disadvantages of this top-down approach are a lack of control of microarchitectural features, poor spatial control over cell seeding, and the inability to direct cell coculture seeding and function [3]. An alternative approach, the bottom-up technique, focuses on designing building blocks with specific microarchitectural features and assembling these blocks into macroscale engineered tissues [3]. While the major advantages of this technique are better control of cell seeding, and microarchitecture than with top-down approaches, one of the major challenges is in creating assembly techniques to make engineered tissues with clinically relevant length scales and mechanical properties.

The cellular microenvironment, such as soluble factors, extracellular matrix (ECM), and cell-cell interactions can control cell behavior in vivo; therefore, improved control over these interactions can better direct the development and function of engineered tissues [4]. Microfabrication technologies have been applied to tissue engineering to mimic the complex in vivo spatiotemporal microenvironment leading to engineered tissues with biomimetic properties and microarchitectures [5]. The bottom-up approach mimics natural tissue structures, such as in the liver, in which repeating functional units arranged in an organized manner [6]. Cell-laden hydrogels are well suited for this process as their biological, physical, and chemical characteristics are similar to many of the tissues of interest [7–11].

For the proposed assembly process, we assemble engineered tissues through control of hydrophilic/hydrophobic interactions of cell-laden hydrogels. First, a biodegradable hydrogel prepolymer is mixed with a photoinitiator. Cell viability of the system can be optimized by the type and the concentration of the photoinitiator [12]. UV light is then passed through a photomask to cross-link the prepolymers and to construct cell-laden building blocks with specific microarchitectures. The UV light passes only through the desired pattern, causing only those regions to polymerize. The building blocks are randomly placed on a hydrophobic surface and are attracted to and move towards each other due to the surface tension on the liquid-air interface [13, 14]. The blocks continue to move towards each other, creating aggregates, the mechanics of which can be directed to some extent by control of external factors, such as the solution and polymer characteristics and agitation [15]. A stir plate or rotary shaker can be used, for example, to improve the movement of the building blocks toward the center of the container directing aggregation. To minimize the surface free energy, the constructed self-assembled cell-laden scaffold remains aggregated on the surface of hydrophobic component [14, 16]. The scaffold is eventually harvested by exposing the system to the UV light for a short time period to polymerize the blocks into one large scale unit.

This method can be used to fabricate cell-laden scaffolds for engineering complex tissues within a range of micro- to mesoscale [13]. Using these techniques it is possible to create engineered tissues on a clinically relevant length scale with precise control over the microarchitecture of the building blocks. In general, it is possible to create centimeter scale tissues with micro/scale resolution. In addition, this system provides significant chemical (mixing specific growth factors within building blocks) [17, 18], biological (encapsulating specific cell types within the pre-polymer) and geometrical (using photo masks with different patterns) control on the final product.

## 7.2 Experimental Design

This procedure is proposed for direct fabrication of self-assembled engineered tissue constructs of clinically relevant length scales with control over the microarchitectural features. In this method cells are encapsulated in hydrophilic, biodegradable polyethylene glycol diacrylate (PEG) hydrogels. These hydrophilic PEG building blocks are randomly placed on the surface of a high-density hydrophobic solution ($CCl_4$) where they will migrate towards each other by surface tension forces to construct an aggregated scaffold. Aggregation of various building blocks leads to the construction of tissue constructs through a secondary UV cross-linking step (Figure 7.1). This method can be performed with minimal necessary expertise or expensive customized equipment. In addition, as the hydrophobic-hydrophilic interface plays a critical role in this process, any material with these characteristics is applicable for this process. For instance, perfluorodecalin could be used in place of $CCl_4$ or methacrylated hyaluronic acid could replace PEG for cell encapsulation. Preliminary experiments in our lab have shown these to be viable alternatives, and there are likely many other solutions and polymers that would work in this system.

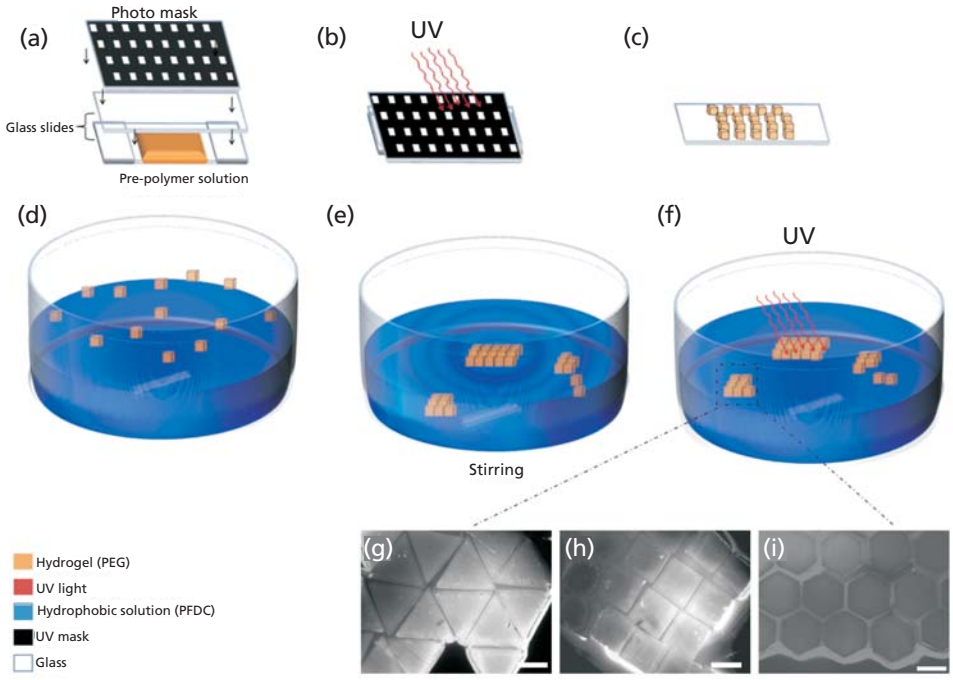


**Figure 7.1** Schematic illustration of proposed self-assembly technique. (a) PEG solution containing cells was placed on top of the OTS treated glass slide between the mask and spacers. (b) UV light exposed through the mask, which gel the PEG-cell solution. (c) The PEG-cell mixture is gelled on top of OTS treated glass. (d) The hydrogel units are randomly placed on the surface of carbon tetrachloride ($CCl_4$. (e) Stirring the solution produced a centripetal force to mimic the scaffold. (f) Aggregates form because of the tendency to reduce the surface free energy of the hydrogel particles. (g–i) Pictures of fabricated hydrogels through the presented methods. Scale bar: 1 mm.

## 7.3 Materials

- Ethanol (Fisher Scientific)
- Polyethylene glycol diacrylate (PEG), mw = 4,000 (Polyscience)
- Irgacure-2959 (I-2959) photoinitiator (Ciba)
- Dulbecco's phosphate-buffered saline (DPBS, GIBCO)
- Ocadecyltrichlorosilane 90%, OTS (Alrich)
- Hexane 95% (Aldrich)
- Photomask with desired design
- Microscope slide, 25 mm × 75 mm × 1 mm (Fisherbrand)
- Microcover glass, 18 mm × 18 mm (VWR)
- UV light source 365 MM source for polyethylene glycol diacrilate gel polymerization

## 7.4 Method

### 7.4.1 Preparation of of 20% poly (ethylene glycol) diacrylate prepolymer

PEG (with molecular weights 1,000 or greater) is a hydrophilic, photopolymerizable hydrogel that has been shown to maintain cell viability in many cell encapsulation applications [19–21]. The first step is to make the prepolymer solution; in this instance we use 20% PEG, but lower percentages may be acceptable for different applications, as could other acrylated polyethelene glycol.

1. Mix 0.2g PEG powder with 0.79-ml DPBS.
2. Add 0.01g photoinitiator to the solution.
3. Use vortex to thoroughly mix the solution.
4. Leave the sample in 80°C oven for 10 to 15 minutes.
5. Remove the sample from the oven and allow sample to reach equilibrium with room temperature (25°C).

Note that the sample is UV sensitive; therefore, it is necessary to either keep the sample covered or contained in a dark glass container at all times following addition of the photoinitiator.

### 7.4.2 Cell preparation (NIH 3T3)

Cell encapsulation in PEG hydrogels can be performed on a wide variety of cells. In this instance we use NIH 3T3 cells; however, any number of cell types could be used. Internal protocols for removing cells from flasks may be used in place of this step.

1. Aspirate the media from cell flask.
2. Based on size of the flask add 10 ml (T-75) or 15 ml (T-175) DPBS to wash reservoir media from cells.
3. Aspirate the PBS from the flask.
4. Add 1× trypsin to the flask, using 5 ml for T-75 or 10 ml for T-175 flasks.
5. Leave the flask in 37°C incubator for 3 to 5 minutes.

6. Rotate the solution a few times in the flask to make sure all the cells are detached.
7. If the majority of cells are detached, add an equal amount of media to the inactive trypsin. Otherwise, repeat steps 5–6 until cells are detached and then proceed with this step.
8. Remove the cell suspension from flask and add to an appropriate sterile centrifuge tube.
9. Centrifuge the solution for 5 minutes to separate the cells; speed = 1,000 rpm.

### 7.4.3 Storage of PEG

PEG is a photosensitive solution; therefore, it is advisable to keep it in colored containers or cover all exposed surfaces with aluminum foil.

1. The prepared PEG solution should be stored at 4°C.
2. It is better to use PEG after reaching equilibrium with room temperature (25°C).

### 7.4.4 Mixing PEG solution with cells

The cells must be mixed in the PEG prepolymer solution prior to polymerization, all while maintaining sterility.

Within a laminar flow hood:

1. Remove media from the cell pellet container.
2. Add appropriate amount of PEG solution to the container to obtain desired cell density.
3. Rotate the PEG solution a few times gently to make sure it is well mixed.

### 7.4.5 Glass OTS treatment

Treating the glass slides with OTS prevents the hydrogels from attaching to the slides upon polymerization with UV, making it easier to harvest the cell-laden gels intact.

1. Wash the glass slide with ethanol two to three times.
2. Mix 0.25-ml OTS in 4.75-ml hexane, to make a 5% OTS solution.
3. Cover the surface of cleaned glass slides with a 5% OTS solution, making sure to mark to which side the treatment was applied.
4. Incubate the glass slides at room temperature for 30 minutes in a chemical hood.
5. Aspirate the 5% OTS solution from the glass slides.
6. Put the slides in an 80°C oven for 2 hours.
7. Remove the sample from the oven.
8. Wash the sample with ethanol.
9. Remove any remaining solution from the surface of the glass slides.

### 7.4.6 Photolithography

To cross-link the cell-laden PEG in the desired shapes, UV light is passed through a photomask. The photomask should be clear in the desired pattern to pass UV light through, and opaque everywhere else. Features as small as 100 $\mu$m can typically be reproduced with good fidelity. The photomask should be printed on a high resolution printer and designed in a CAD program—typical office printers do not often have sufficient

resolution to produce these transparencies. Note that to maintain sterility, all components (slides, photomasks, and so forth) must be handled either with sterile gloves, sterilized forceps, or something similar.

Within a laminar flow hood:

1. Sterilize the OTS-treated microscope slides and cover slips by spraying with 70% ethanol and allow to dry.
2. Pipette a 35-$\mu$l PEG-cell solution on treated slide surface.
3. Adjust the desired height by stacking glass cover slips on top of the slide glass on each side of the PEG-cell solution. (Each cover slip typically has a thickness of 150 $\mu$m, but this can vary. Read manufacturers specifications carefully prior to use.)
4. Place a clean glass slide on top of the cover slips, such that the PEG-cell solution is located between the two slides and on the OTS-treated surface of both.
5. Place the desired photo mask on top of the uppermost treated slide.
6. Place entire assembly into a sterile Petri dish.
7. Sterilize the UV area with ethanol.
8. Shine the UV through the assembly in the Petri dish to polymerize the PEG cell solution.
9. Optimized conditions for UV exposure:
   - Height of 78 mm;
   - UV power of 12.5 $\omega/cm^2$;
   - Time = 27 seconds. It is important to have some prepolymer left on each sample.
10. Return to hood.
11. Remove the Petri dish cover and then the microscope slide glass.
12. Wash away the remaining ungelled PEG-cell solution with DPBS.

### 7.4.7 Aggregation process

To aggregate the cell-laden particles it is necessary to add them to an appropriate volume of dense, hydrophobic solution in a sterile container in a laminar flow hood or a sealed container.

Within a laminar flow hood:

1. Adjust the height of solution in the sterile container (in this case, 3 cm).
2. The shape of the vertex created due to stirring speed is a direct function of the height of carbon tetrachloride. Therefore the suggested numbers are only applicable for a 3-cm height.
3. Place the cell-laden hydrogel blocks randomly on the surface of the carbon tetrachloride. Cover before using stir plate outside of hood.
4. Adjust the stirring speed to find the optimized speed (in our case 200 rpm). One minute should be enough time for particles to aggregate. Do not keep the particles for more than 10 minutes in the $CCl_4$ solution to maintain viability.
5. Turn off the stirring motion and allow the construct to become motionless.
6. Place the construct under UV light to initiate secondary cross-linking (to freeze particles in the aggregated form) for 5 seconds.
7. Return to hood.
8. Using a glass slide, remove the particles from carbon tetrachloride solution.

### 7.4.8 Data analysis

To quantify the particle aggregation, we introduced an *aggregation factor* (AF). The aggregation factor was calculated and normalized based on the surrounding area of the aggregated particles as: $A_f = 1 - a_r/a_s$, where $A_f$ is the aggregation factor, $a_r$ is the resultant surrounding area, and $a_s$ is the surrounding area of all aggregated particles while they are detached.

## 7.5 Anticipated Results

Following these steps, the final product will be a 3D tissue-like construct composed of individual building blocks. However to optimize the system, it is necessary to characterize the effects of the potential cell toxins such as $CCl_4$ and UV exposure. To characterize these effects, cell viability was tested in a study designed to simulate each step of the procedure using the live/dead assay (Figure 7.2). Cell viability is roughly 95% in control cells mixed in the PEG prepolymer only, 87% in cells/PEG exposed to UV light for 27 seconds, 80% following a 1-minute agitation in $CCl_4$, and 52% following secondary UV exposure in $CCl_4$ for 5 seconds. As the end goal is to create engineered tissues with controlled microarchitectural features, improvement of cell viability is critical to achieving the final goal. Recently published results in our group have demonstrated excellent viability after 7 days of culture by substituting $CCl_4$ with perfluorodecalin [22]. Investigation into use of other dense, hydrophobic solutions with improved cell compatibility, as well as optimization of the techniques to minimize exposure to potential cell toxins, could potentially further optimize this technique.

Building blocks with any desired geometry can be fabricated in the proposed platform; however, symmetric particles are more favorable than asymmetric ones. Increasing the thickness of the building blocks increases the aggregation factor by increasing of the surface energy, but also would affect the UV exposure time, potentially affecting the viability. Moreover, increasing the number of the particles in each experiment can improve the hydrogel aggregation process. The number of the particles and the thickness of the building blocks are linearly related to the aggregation factor; however, the aggregation factor can also be optimized by the stirring speed. The relation between the speed and the aggregation factor is depicted in Figure 7.3.

## 7.6 Discussion and Commentary

Current approaches to fabricate 3D tissues are typically based on seeding cells on 3D scaffolds with random pore structures, leaving little control over the cellular microenvironment and structure. Cell-cell and cell-ECM interactions can direct the morphogenesis to control the cell proliferation and construct a functional tissue, making control of the microarchitecture paramount to controlling tissue morphogenesis. To control the complex spatiotemporal micro-environment and mimic the ECM, microfabrication technologies have been applied to in vitro systems to encapsulate cells in specific microarchitectural arrangements. Tissue engineering methods with the capability of controlling the microarchitecture are desired to fabricate tissue structures with controlled cell behavior. This was the main motivation to design the

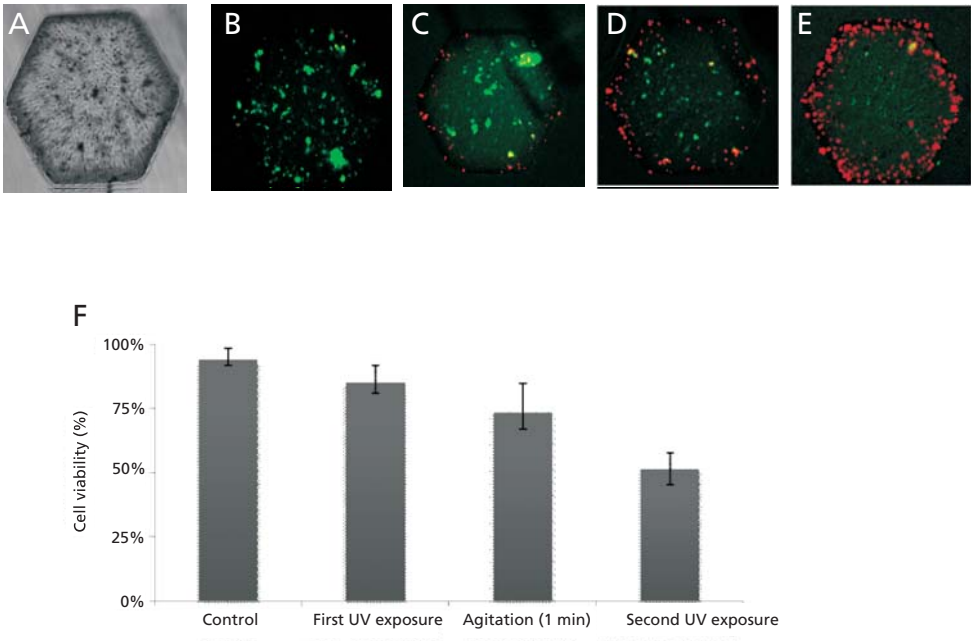


**Figure 7.2** Cell viability following UV and $CCl_4$ exposure. (a) Phase contrast image of individual cell-laden microgel. Live/dead images of cell-laden microgels (b) prior to exposure, (c) following the first UV exposure, (d) after agitation in $CCl_4$, and (e) following the second UV exposure. (f) Quantification of live/dead imaging demonstrating the percentage of living cells.

proposed technique with the capability of fabrication of cell-laden tissue constructs by applying 2D self-assembly on a surface.

Employing surface tension as a driving force enables application of this method in a broad range from the nano- to the mesoscale [13]. Furthermore, control of external factors to the system, such as the centripetal forces caused by stirring, can better control the macrostructure of the final construct. The final product of this process will be a cell-laden tissue layer, composed of building blocks potentially consisting of multiple cell types and growth factors with specific microarchitectural features specific to the desired tissue type. The final shape of the fabricated layer is not fully predictable; however, in order to increase the control over the final shape of the construct, a series of system characteristics such as thickness of building blocks, PEG molecular weight, initial number of particles, and the stirring speed were optimized.

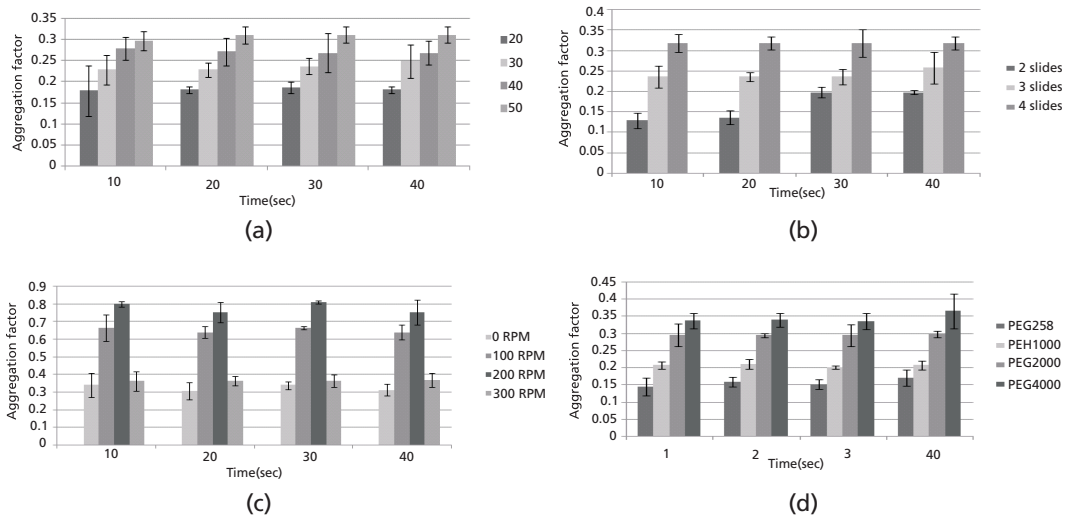


**Figure 7.3** Characterization of the aggregation factor. (a) Effect of initial number of particles on aggregation factor. (b) Effect of building block thickness on aggregation factor. (c) Effect of stirring speed on aggregation factor. (d) Effect of PEG molecular weight on aggregation factor.

**Troubleshooting Table**

| Problem | Explanation | Potential Solution |
|---|---|---|
| Effect of UV exposure on viability. | Excess UV is lethal for cells. | The overall UV exposure time should not be more than 30 seconds. |
| Poor aggregation or dissociation of aggregates. | Shape of vortex produced by stirring should be optimized. | Adjust stirring speed and/or height of CCl4. |
| No aggregation. | Dust may disturb the surface tension. | It is recommended to run the process under the hood. |
| Poor cell viability, hydrogel drying. | It is possible for the hydrogels to be dehydrate, affecting the cell viability. | It is highly recommended to manage the process such that it will be done within 10 minutes from cell capsulation to second cross-linking. |

## 7.7 Application Notes

This chapter describes the fabrication of 3D tissue-like structures constructed by the self-assembly of cell-laden hydrogels where cell-laden blocks are aggregated by enhancing the hydrophilic-hydrophobic interactions. The proposed technique is an enabling technology to fabricate tissues with repeated building blocks [23] such as the liver, skin, and bladder [24]. This method can potentially be used for in vitro and in vivo research for tissue engineering.

## 7.8 Summary Points

1. We developed a self-assembly process to fabricate cell-laden tissue-like constructs, which can be applied to fabricate tissues with repeated units.

2. The self-aggregated scaffolds were optimized as a function of PEG molecular weight, thickness of building blocks, number of particles, and stirring speed.
3. The cell viability was quantified and optimized at different stages as a function of different factors, which lead to the cell death.

## Acknowledgments

We thank Masoud Khabiri, Dr.Yanan Du, and Dr. Seunghwan Lee who helped us on this work. This work was supported by the National Institutes of Health (EB007249; DE019024; HL092836), the NSF CAREER award, the Institute for Soldier Nanotechnology, and the U.S. Army Corps of Engineers.

### References

[1] Koh, H. K., et al., "A Statewide Public Health Approach to Improving Organ Donation: The Massachusetts Organ Donation Initiative," *American Journal of Public Health*, 2007, pp. 30–36.

[2] Langer, R., and J. Vacanti, "Tissue Engineering," *Science*, Vol. 260, No. 5110, 1993, pp. 920–926.

[3] Nichol, J. W., and A. Khademhosseini, "Modular Tissue Engineering: Engineering Biological Tissues from the Bottom Up," *Soft Matter*, Vol. 5, 2009, pp. 1312–1319.

[4] Hamilton, D., K. Wong, and D. Brunette, "Microfabricated Discontinuous-Edge Surface Topographies Influence Osteoblast Adhesion, Migration, Cytoskeletal Organization, and Proliferation and Enhance Matrix and Mineral Deposition In Vitro," *Calcified Tissue International*, Vol. 78, No. 5, 2006, pp. 314–325.

[5] Khademhosseini, A., et al., "Micromolding of Photocrosslinkable Hyaluronic Acid for Cell Encapsulation and Entrapment," *J. Biomed. Mater. Res. A*, Vol. 79, No. 3, 2006, pp. 522–532.

[6] Tsang, V. L., et al., "Fabrication of 3D Hepatic Tissues by Additive Photopatterning of Cellular Hydrogels," *FASEB. J.*, Vol. 21, No. 3, 2007, pp. 790–801.

[7] Ringel, R. L., et al., "The Application of Tissue Engineering Procedures to Repair the Larynx," *Journal of Speech, Language & Hearing Research*, 2006, pp. 194–208.

[8] Allan, S. H., "Hydrogels for Biomedical Applications," *Annals of the New York Academy of Sciences*, Vol. 944, 2001, pp. 62–73.

[9] Dong-An, W., et al., "Multifunctional Chondroitin Sulphate for Cartilage Tissue Biomaterial Integration," *Nature Materials*, 2007, pp. 385–392.

[10] Ifkovits, J. L., and J. A. Burdick, "Review: Photopolymerizable and Degradable Biomaterials for Tissue Engineering Applications," *Tissue Eng.*, Vol. 13, No. 10, 2007, p. 2369.

[11] Lee, K. Y., and D. J. Mooney, "Hydrogels for Tissue Engineering," *Chemical Reviews*, Vol. 101, No. 7, 2001, pp. 1869–1880.

[12] Fisher, J. P., et al., "Photoinitiated Polymerization of Biomaterials," *Annual Review of Materials Research*, Vol. 31, No. 1, 2001, pp. 171–181.

[13] Whitesides, G. M., and B. Grzybowski, "Self-Assembly at All Scales," *Science*, Vol. 295, No. 5564, 2002, pp. 2418–2421.

[14] Kralchevsky, P. A., et al., "Capillary Meniscus Interaction Between Colloidal Particles Attached to a Liquid-Fluid Interface," *Journal of Colloid and Interface Science*, Vol. 151, 1992, pp. 79–93.

[15] Breen, T. L., et al., "Design and Self Assembly of Open, Regular, 3D Mesostructures," *Science*, Vol. 284, 1997, pp. 848–851.

[16] Kralchevsky, P. A., et al., "Energetical and Force Approaches to the Capillary Interactions Between Particles Attached to a Liquid-Fluid Interface," *J. Colloid Interface Sci.*, Vol. 155, 1993, pp. 420–437.

[17] Peppas, N. A., et al., "Hydrogels in Pharmaceutical Formulations," *European Journal of Pharmaceutics and Biopharmaceutics*, Vol. 50, No. 1, 2000, pp. 27–46.

[18] Byrne, M. E., K. Park, and N. A. Peppas, "Molecular Imprinting Within Hydrogels," *Science*, Vol. 54, No. 1, 2002, pp. 149–161.

[19] Revzin, A., et al., "Fabrication of Poly(Ethylene Glycol) Hydrogel Microstructures Using Photolithography," *Langmuir*, Vol. 17, 2001, pp. 5440–5447.

[20] Koh, W. G., A. Revzin, and M. V. Pishko, "Poly(Ethylene Glycol) Hydrogel Microstructures Encapsulating Living Cells," *Langmuir*, Vol. 18, No. 7, 2002, pp. 2459–2462.

[21] Khademhosseini, A., et al., "Molded Polyethylene Glycol Microstructures for Capturing Cells Within Microfluidic Channels," *Lab Chip*, Vol. 4, No. 5, 2004, pp. 425–430.

[22] Zamanian, B., et al., "Interface Directed Self-Assembly of Cell-Laden Microgels into Macroscale Engineered Tissues," Small, 2010. in revision.

[23] Khademhosseini, A., J. P. Vacanti, and R. Langer, "Progress in Tissue Engineering," *Sci. Am.*, Vol. 300, No. 5, 2009, pp. 64–71.

[24] Costanzo, L., *Physiology,* 3rd ed, Philadelphia, PA, Saunders, 2006.