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Method of Bottom-Up Directed Assembly of Cell-Laden Microgels

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Abstract—The paper describes a protocol to fabricate cell-laden microgel assemblies with pre-defined micro-architecture and complexity by a bottom-up approach, which can be used for tissue engineering applications. The assembly process was driven by hydrophobic effect in the water/oil interface. By agitating hydrophilic microgels in hydrophobic medium, the shape-controlled microgel units assemble in an organized manner to locally minimize the interaction free energy (the surface area exposed to the oil). The assembly process was shown to be controlled by several parameters, such as external energy input, surface tension, and microgel dimensions. This assembly approach was used to build multi-component cell-laden constructs by assembling microgel building blocks and performing a secondary cross-linking reaction. This bottom-up approach for the directed assembly of cell-laden microgels offers a scalable method to fabricate 3D tissue constructs with biomimetic structure.

Keywords—Bottom-up, Tissue engineering, Assembly, Hydrogel.

ABBREVIATIONS

3D	3-Dimensional
DPBS	Dulbecco's phosphate buffered saline
MW	Molecular weight
PEG	Polyethylene glycol
UV	Ultra violet

INTRODUCTION

This paper describes in detail the procedure for a bottom-up approach to assemble cell-laden microscale hydrogels (microgels) for fabrication of 3D tissue constructs.[3] Bottom-up approaches build larger tissue constructs by the assembly of smaller cell-laden building blocks (i.e. microgels), which mimics the living tissue architecture from repeating functional units (i.e. islet, nephron or sinusoid).[2,4] Thus far, bottom-up assembly of cell-laden microgels has been gaining increasing attention in the tissue engineering research, with numerous approaches developed including random assembly,[6] manual manipulation,[8] multi-layer photo-patterning,[5,7] and microfluidic-directed assembly.[1] Random assembly of microgel modules has the advantage of being rapid and simple, but lacks control over the final structure of the microgel assembly; manual manipulations are relatively slow processes and not scalable, multi-layer photo-patterning and microfluidic-directed assembly are able to create highly sophisticated microgel assembly architectures, but requires longer operational time and sophisticated equipments.

The bottom-up assembly process presented here aims to direct the assembly of cell-laden microgels in a simple and highly scalable manner. The assembly process is driven by the 'hydrophobic effect'—the thermodynamic tendency of multiphase liquid–liquid systems to minimize the surface free energy between the phases (Fig. 1). Cell-laden microgels with defined sizes and shapes that were fabricated by photolithography were transferred into a hydrophobic mineral oil phase, and assembled with tunable micro-architecture upon application of a controlled agitation force. The cell-laden microgel assemblies could be further stabilized and harvested from the mineral oil for culturing in aqueous medium after a secondary cross-linking step. By assembling rectangular-shaped microgels, it was possible to control the overall dimensions and architecture of the assembly. To demonstrate the utility of this approach for generating more complex and 'directed' structures, a 'lock-and-key' design for the microgel shapes was used to control the relative position of two different types of microgels in the final assembly.

This approach requires the use of a hydrophobic phase such as mineral oil, which requires that living

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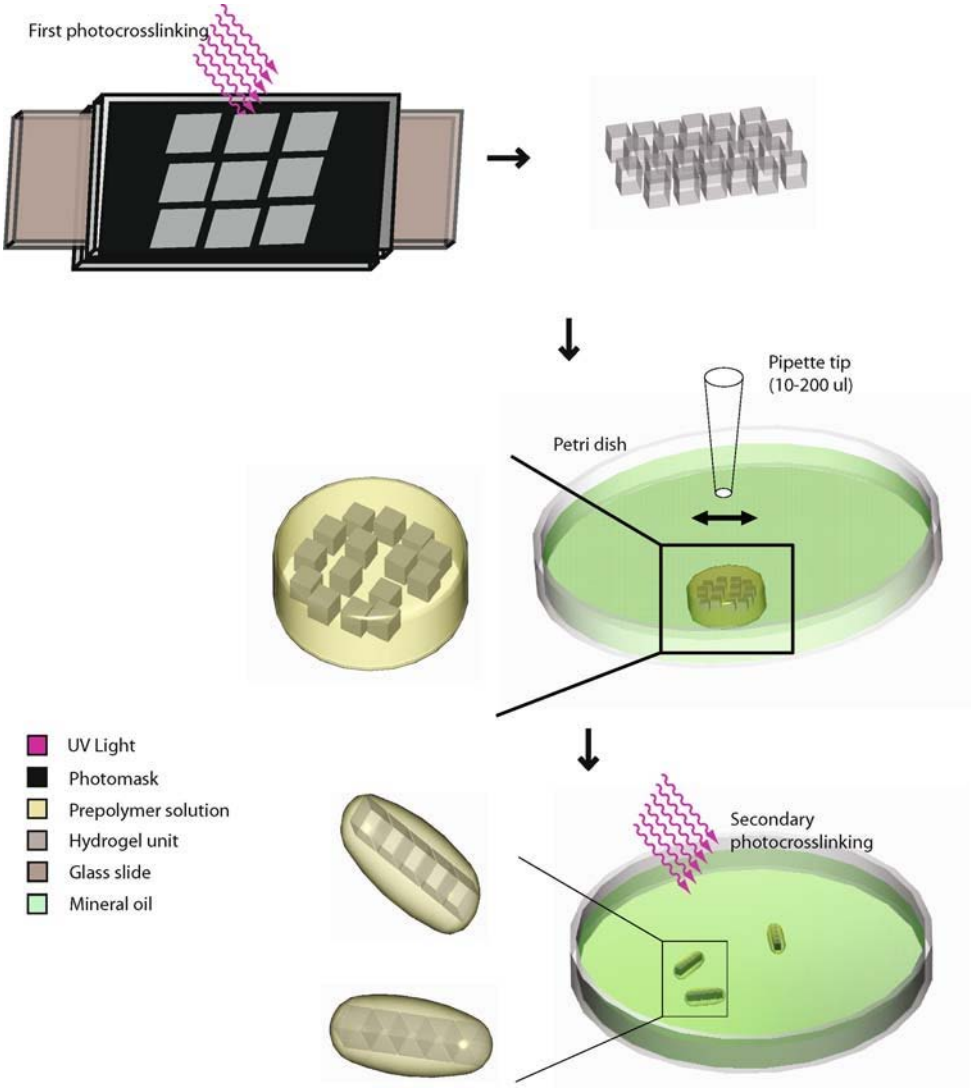

FIGURE 1. Schematic representation of the microgel assembling. The steps of assembly process of microgel units are as follows: Synthesizing the microgel units by photolithography, transferring them into a petri dish filled with mineral oil, applying mechanical agitation through manual movement of the pipette-tip back and forth, exposing the microgel assemblies to UV-light for secondary crosslinking.

cells be encapsulated in microgels to prevent direct exposure to the hydrophobic oil phase during the assembly procedure. In addition, it is still challenging to control the assembly three-dimensionally and achieve assemblies with uniform shapes (i.e. linear, branched and random assemblies were formed from assembling of square-shaped microgels).

The bottom-up approach assembly of microgels with defined 3D structures is a promising approach for engineering tissue constructs at large scale, which mimic the complexity of living tissues and opens a paradigm for directing the assembly of other mesoscale materials. By fabricating microgel building blocks with more complex geometries and properties and adoption of the secondary crosslinking, this approach can be potentially used to build biomimetic higher-order tissue construct that may be difficult and time-consuming to fabricate by using traditional tissue engineering methods. This protocol paper is expected to facilitate the application and further improvement of this approach.

REAGENTS

- Poly(ethylene glycol)-dimethacrylate polymer 1000 (Polysciences, Inc.; cat. no. 15178)
- Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, cat. no. 14190)
- 1% photoinitiator (2-hydroxy-1-[4-(hydroxy-ethoxy) phenyl]-2-methyl-1-propanone) (Ciba Chemicals, Irgacure 2959)

- Mineral Oil (CVS Pharmacy)
- Tween 20 (surfactant) (Sigma, cat. no. P-5927)
- FITC-dextran (MW = 2000 kDa) (Sigma, cat. no. FD2000S)
- Rhodamine-dextran (MW = 10 kDa) (Sigma, cat. no. R8881)
- Nile red (MW = 317 kDa) (Sigma, cat. no. N3013)
- Green fluorescent microbeads (1% solid, D = 5 μm) (Duke Scientific, G0500)
- NIH 3T3 mouse fibroblasts
- Dulbecco's Modified Eagle Medium (Gibco, cat. no. 11965)
- 10% FBS (to supplement DMEM) (Gibco, cat. no. 26140)
- 0.5% Trypsin-EDTA 10x (Gibco, cat. no. 15400)
- Live/Dead dyes: Calcein AM and Ethidium Homodimer-1 (Molecular Probes, cat. no. L3224)
- PKH26 Red Fluorescent Cell Linker (Sigma, cat. no. PKH26GL-1KT)

EQUIPMENT

- Photomasks with different patterns (i.e. rectangular or lock-and-key) designed using AutoCAD and printed on transparencies with 20,000-dpi resolution (CAD/Art Services)
- Micro cover glasses (150 μm thick, 18 × 18 mm) (VWR, cat. no. 48366 045)
- UV light (The OmniCure® S2000 UV/Visible Spot Curing System)
- 60 × 15-mm dish (Fisher Scientific, cat. no. 430589)
- 10 μL pipette tips
- 15-mL tubes (BD Biosciences, cat. no. 352096)
- Vortexer
- Centrifuge
- Incubator (5% CO_2 at 37 °C)
- Microscope (with 4× and 10× objective lenses)

REAGENT SETUP

Preparation of Prepolymer Solution

Dissolve 20% (wt/wt) poly(ethylene glycol)-methacrylate polymer in Dulbecco's Phosphate Buffered Saline. Add 1% (wt/wt) photoinitiator before UV polymerization.

Preparation of Cells for Encapsulation Within Prepolymer Solution

Add 1× trypsin to cells in the flask and resuspend the cells in the prepolymer solution at a concentration of 1 × 10^7 cells/mL.

Preparation of Live/Dead Dyes

Add 2 μL of Calcein AM and 0.5 μL of Ethidium homodimer to 1 mL of DPBS.

PROCEDURE

Prepolymer Solution Preparation • Timing 15 min

1. Mix 20% (w/w) of polyethylene glycol (PEG) with 80% (w/w) Dulbecco's Phosphate Buffered Saline (DPBS). Vortex until the PEG fully dissolves.
2. Add 1% of photoinitiator (2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone) to this solution. Vortex until the photoinitiator fully dissolves.

Note Dissolving the PEG first is essential, as the photoinitiator is difficult to dissolve otherwise.

Microgel Fabrication • Timing 3 min/Slide

1. Design photomasks to control the desired shape of the microgels. Masks can be designed using programs such as Macromedia Freehand or AutoCAD and printed using a high resolution printer (with minimum 20,000-dpi resolution).
2. Place a drop of prepolymer solution (with the volume of 30 μL) in the middle of a base glass slide, then place spacer slides on the opposite of the base slide to control the height of the gels (the thickness of one spacer slide is 150 μm). To adjust the height of the gels, different numbers of spacer slides can be combined. The size of the drop should be adjusted to ensure prepolymer does not spill over the edge when a cover glass slide is placed above the solution.
3. Shine UV light on the device to induce gel formation. The exposure time must be adjusted depending on the size of the features in the gel. For a 400 × 400 × 150 μm microgel, a UV exposure of 12.4 mW cm^{-2} at 360–480 nm for 30 s was sufficient for formation.
4. Separate the top and bottom slides to allow for microgel collection.

Note If the gels will not be used right away, they should be kept under DPBS to avoid adherence to the glass slides.

Microgel Assembly • Timing 10 min

1. Fill a 60 × 60 × 15 mm Petri dish with 6ml of mineral oil.
2. Collect the microgels into a small cluster. Dry all excess liquid and then drop a minimal amount of prepolymer solution on the microgels (~5 μL).

3. Transfer the microgels to the mineral oil filled dish.
4. Sketch straight lines with a thin pipette tip (1–200 μL) through the cluster of microgels. Continue this for 60 s with an agitation rate of 36 cm s^{-1} (Corresponding to Reynolds number of 3).

Note If lines are sketched too slowly, ordered assembly will not occur.

5. Place the entire dish under UV light again to stabilize the structures. A shorter time can be used for this stabilization (using the previously mentioned conditions, 4 s is sufficient for stabilization).

Note If an excess of hydrophilic liquid remains in the gel cluster, assembly will not occur as effectively. If microgels do not assemble properly, try drying the cluster more.

Cell-Laden Microgel Assembly • *Timing 60 min*

1. Isolate a sufficient number of cells for all experiments. Typically, a cell density of 1×10^7 cells/mL of prepolymer solution was used.
2. Obtain a cell pellet by centrifuging at 800 rpm and remove excess cell medium. Re-suspend the pellet in prepolymer solution.
3. Repeat the '*Microgel fabrication*' and '*Microgel assembly*' procedures. Ensure the solution and cells are well mixed by pipetting the mixture up and down several times prior to the fabrication of each slide.

Note If solution is not well mixed, cells will sink to the bottom of the solution and hence not be well distributed in the gels.

Note Avoid making bubbles in the solution by keeping the pipette tip submerged when pipetting up and down.

4. After secondary UV exposure, remove excess mineral oil from the dish with a pipette. Wash the dish with cell culture medium 3 times to remove more oil and then submerge the structures in medium.

Note Take caution and avoid microgels when aspirating the mineral oil and culture medium.

Timing

Prepolymer solution preparation (15 min)
Microgel fabrication (3 min/slide)
Microgel assembly (10 min)
Cell-laden microgel assembly (60 min)

Troubleshooting.

Problem	Possible reason	Suggested solution
Microgels do not form	Mask size is too small	Increase the feature sizes
	Insufficient UV exposure	Increase UV time
Microgels were broken	Agitation too harsh	Decrease Reynolds number by decreasing agitation rate
	Pipette tip too sharp	Use a larger pipette tip
Failure of secondary crosslinking	Prepolymer was washed away prior to second crosslinking	Add a small amount of prepolymer to the microgel cluster prior to agitation
	Not enough secondary UV exposure	Increase secondary UV exposure time
Cells in the gel are dead	Excessive UV exposure	Decrease first and/or second UV exposure (though the gels and aggregates must still be formed)

ANTICIPATED RESULTS

By using the current protocol, microgels with different dimensions from 200 to 1000 μm with 200 μm increments (for rectangular microgels) and different shapes (lock & key) could be assembled. In case of rectangular microgel assemblies made with microgels of different sizes, it was observed that the average length of the linear microgel assemblies has a direct relation with the aspect ratios of the microgels. To be more specific, the obtained aspect ratio of microgel assemblies is in proximity of 1 (Fig. 2a).

In order to show the feasibility of this technique for generation of more sophisticated and 'directed' structures, the microgel shapes was designed in a 'lock-and-key' pattern. The 'lock-and-key' design enables us to locate two different types of microgels in a desired location in the final assembly (Fig. 2b).

To stabilize the interaction between assembled microgel structures (Fig. 2c), a secondary crosslinking was applied. This step was performed after the formation of microgel assemblies. It is also noticeable that the residual prepolymer solution surrounding the individual microgels prior to agitation was necessary for the success of the secondary crosslinking. As a demonstration of the use of the micro-scale hydrogel assembly process developed here for biological applications, NIH-3T3 fibroblasts were encapsulated within the individual microgels and assembled into linear structures (Fig. 3a). A high fraction of the cells remained viable immediately

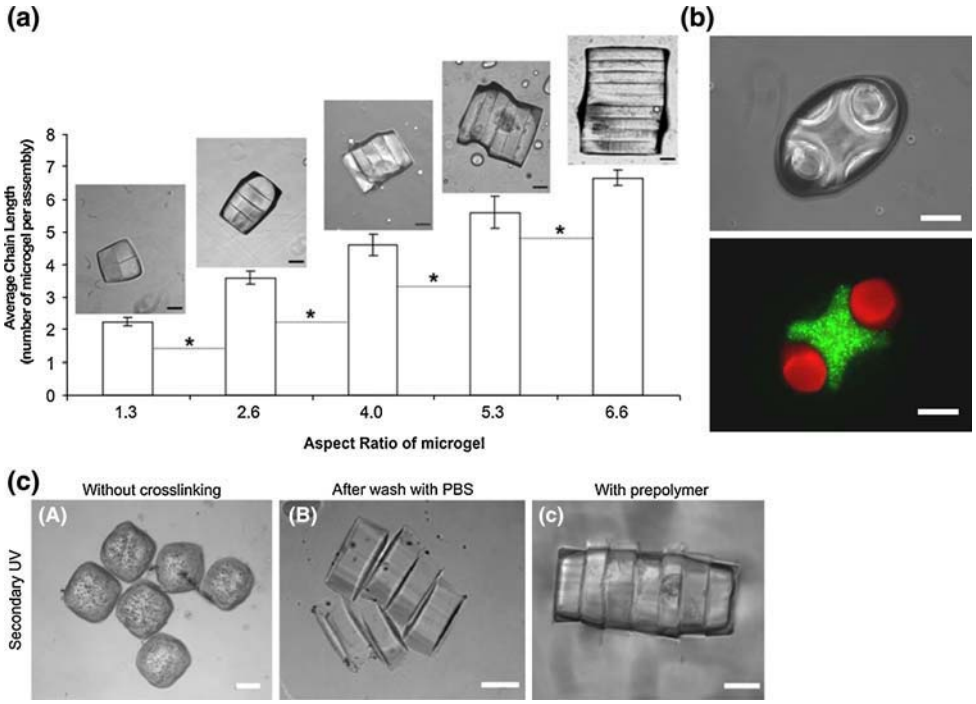

FIGURE 2. Microgel assembly formation and stabilization. (a) Phase images and average chain length of the microgel assemblies of rectangular microgels with different dimensions; (b) fluorescence and phase images of the directed assembly of lock-and-key shaped microgels; (c) secondary crosslinking for stabilizing the microgel assemblies. The stages of secondary crosslinking are as follows: (A) dissociating microgel assemblies by removing mineral oil and adding culture medium (*note*: secondary crosslinking is not applied); (B) applying secondary crosslinking on microgel assemblies in culture medium for stabilization purposes; (C) removing residual pre-polymer after secondary crosslinking polymer (all scale bars are representing 200 μm). Adapted from Ref. 3 with permission.

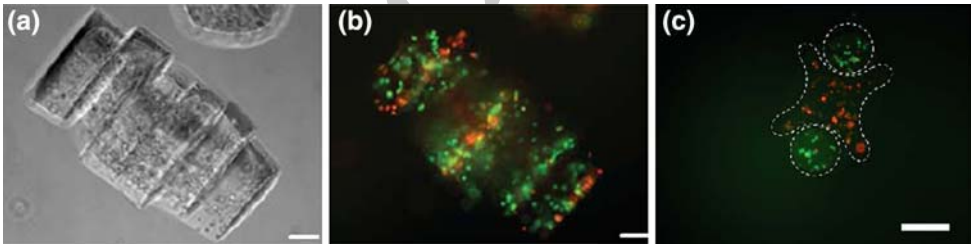

FIGURE 3. Encapsulated cells in microgel assemblies: (a) cell-laden (NIH-3T3) microgel assemblies are shown after first and secondary crosslinking in phase contrast and fluorescence image form for revealing morphology; (b) cell viability quantification after each step in the process of assembling microgels; (c) fluorescence image to show the microgel assembly composed of cross-shaped microgel (containing red-stained cells) and rod-shaped microgels (containing green-stained cells), (scale bars are 100 μm). Adapted from Ref. 3 with permission.

(Fig. 3b). We further demonstrated the applications of the lock-and-key directed assembly for generating cellular co-cultures.

Two different cell types stained by red and green cell tracker respectively were encapsulated in cross-shaped or rod-shaped microgels (Fig. 3c) and assembled by this approach. Microscale tissue constructs composed of two cell types were fabricated, which can be readily used as co-culture system.

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REFERENCES

[1]Chung, S. E., W. Park, S. Shin, S. A. Lee, and S. Kwon. Guided and fluidic self-assembly of microstructures using railed microfluidic channels. *Nat. Mater.* 7:581–587, 2008.

[2]Costanzo, L. Physiology, 3rd edn. Saunders, 2006.

[3]Du, Y., E. Lo, S. Ali, and A. Khademhosseini. Directed assembly of cell-laden microgels for fabrication of 3D tissue constructs. *Proc. Natl. Acad. Sci. USA* 105:9522–9527, 2008.

[4]Khademhosseini, A., and R. Langer. Microengineered hydrogels for tissue engineering. *Biomaterials* 28:5087–5092, 2007.

[5]Liu Tsang, V., A. A. Chen, L. M. Cho, K. D. Jadin, R. L. Sah, S. DeLong, J. L. West, and S. N. Bhatia. Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. *FASEB J* 21:790–801, 2007.

[6]McGuigan, A. P., and M. V. Sefton. Vascularized organoid engineered by modular assembly enables blood perfusion. *Proc. Natl. Acad. Sci. USA* 103:11461–11466, 2006.

[7]Nahmias, Y., R. E. Schwartz, C. M. Verfaillie, and D. J. Odde. Laser-guided direct writing for three-dimensional tissue engineering. *Biotechnol. Bioeng.* 92:129–136, 2005.

[8]Yeh, J., Y. Ling, J. M. Karp, J. Gantz, A. Chandawarkar, G. Eng, J. Blumling, 3rd, R. Langer, and A. Khademhosseini. Micromolding of shape-controlled, harvestable cell-laden hydrogels. *Biomaterials* 27:5391–5398, 2006.