Generation and Recovery of β-cell Spheroids From Step-growth PEG-peptide Hydrogels

Asad Raza, Chien-Chi Lin
Department of Biomedical Engineering, Engineering, Purdue School of Engineering and Technology, Indiana University - Purdue University at Indianapolis

Correspondence to: Chien-Chi Lin at lincc@iupui.edu

URL: http://www.jove.com/video/50081
DOI: doi:10.3791/50081

Keywords: Biomedical Engineering, Issue 70, Bioengineering, Tissue Engineering, Cellular Biology, Molecular Biology, Biomaterials, beta cells, β-cell, PEG, PEG-peptide hydrogels, hydrogel, MIN6, polymers, peptides, spheroids, pancreas

Date Published: 12/6/2012
Citation: Raza, A., Lin, C. Generation and Recovery of β-cell Spheroids From Step-growth PEG-peptide Hydrogels. J. Vis. Exp. (70), e50081, doi:10.3791/50081 (2012).

Abstract

Hydrogels are hydrophilic crosslinked polymers that provide a three-dimensional microenvironment with tissue-like elasticity and high permeability for culturing therapeutically relevant cells or tissues. Hydrogels prepared from poly(ethylene glycol) (PEG) derivatives are increasingly used for a variety of tissue engineering applications, in part due to their tunable and cytocompatible properties. In this protocol, we utilized thiol-ene step-growth photopolymerizations to fabricate PEG-peptide hydrogels for encapsulating pancreatic MIN6 β-cells. The gels were formed by 4-arm PEG-norbornene (PEG4NB) macromer and a chymotrypsin-sensitive peptide crosslinker (CGGYC). The hydrophilic and non-fouling nature of PEG offers a cytocompatible microenvironment for cell survival and proliferation in 3D, while the use of chymotrypsin-sensitive peptide sequence (CGGY[C, arrow indicates enzyme cleavage site, while terminal cysteine residues were added for thiol-ene crosslinking]) permits rapid recovery of cell constructs forming within the hydrogel. The following protocol elaborates techniques for: (1) Encapsulation of MIN6 β-cells in thiol-ene hydrogels; (2) Qualitative and quantitative cell viability assays to determine cell survival and proliferation; (3) Recovery of cell spheroids using chymotrypsin-mediated gel erosion; and (4) Structural and functional analysis of the recovered spheroids.

Video Link

The video component of this article can be found at http://www.jove.com/video/50081/

Introduction

Hydrogels are hydrophilic crosslinked polymers with exceptional potential as scaffolding materials for repairing and regenerating tissues.1-3 The high water content of hydrogels permits easy diffusion of oxygen and exchange of nutrients and cellular metabolic products, all of which are crucial to maintaining cell viability. In addition, hydrogels are excellent carriers for controlled release and cell delivery due their high tunability.2 Synthetic hydrogels such as those prepared from poly(ethylene glycol) (PEG) are increasingly used in tissue engineering applications, largely due to their cytocompatibility, tissue-like elasticity, and high tunability in material physical and mechanical properties.4,6

Although a commonly used hydrogel platform, studies have shown that PEG diacrylate (PEGDA) hydrogels formed by chain-growth photopolymerizations have a tendency to damage encapsulated cells during network crosslinking and in situ cell encapsulation.7 The cellular damage was largely attributed to radical species generated by the photoinitiator molecules, which propagate through the vinyl groups on PEGDA to crosslink polymer chains into hydrogels. Unfortunately, these radical species also cause stresses and cellular damage during cell encapsulation, especially for radical-sensitive cells such as pancreatic β-cells.8-10 In order to obtain a higher mesh size for better diffusion and cell survival, higher molecular weights PEGDA are often used for cell encapsulation. This, however, compromises polymerization kinetics and causes sub-optimal gel biophysical properties.7,11,12 In addition to the above mentioned disadvantages, it is very difficult to recover cell structures from PEGDA hydrogels due to the heterogeneity and non-degradable nature of the crosslinked networks. While protease-sensitive peptides can be incorporated into PEG macromer backbone to render the otherwise inert PEGDA hydrogels sensitive to enzymatic cleavage, the conjugation often uses expensive reagents and the resulting networks still contain high degree of heterogeneity due to the nature of chain-growth polymerization.13-15

Recently, PEG-peptide hydrogels formed via step-growth thiol-ene photopolymerization have been shown to exhibit preferential properties for cell encapsulation over hydrogels formed by chain-growth photopolymerization.7 The superior gelation kinetics of thiol-ene hydrogels is attributed to the ‘click’ nature of reaction between thiol and ene functionalities. Compared to chain-growth polymerization of PEGDA, thiol-ene reaction is less oxygen inhibited which results in faster gelation rate.16,17 Thiol-ene hydrogels also have higher polymerization efficiency and better gel biophysical properties compared to chain-growth PEGDA hydrogels,7,18 which results in limited cellular damage caused by radical species during photopolymerization.
Previously, thiol-ene hydrogels formed by 4-arm PEG-norbornene (PEG4NB) macromer and bis-cysteine containing peptide crosslinkers, such as protease-sensitive peptides have been utilized for cell encapsulation. High tunability of PEG hydrogel networks offers a flexible and controllable 3D microenvironment for investigating cell survival and activity, while the use of protease-sensitive peptide sequence provides a mild way for recovery of cell constructs formed naturally within hydrogels. In this protocol we utilize step-growth photopolymerized thiol-ene hydrogels fabricated using 4-arm PEG-norbornene (PEG4NB) and a chymotrypsin-sensitive peptide crosslinker (CGGY↓C) for the encapsulation of MIN6 β-cells. This protocol systematically elaborates techniques for studying the survival, proliferation and spheroid formation of MIN6 β-cells in thiol-ene hydrogels. We further provide method for β-cell spheroid recovery and biological characterization of recovered spheroids.

**Protocol**

**A. Macromer and Peptide Synthesis**

1. Synthesize 4-arm PEG-norbornene (PEG4NB) and photoinitiator Lithium arylphosphanate (LAP) using established protocols.18,19
2. Synthesize bis-cysteine containing chymotrypsin-sensitive peptide CGGY↓C (arrow indicates chymotrypsin cleavage site) using standard solid phase peptide synthesis in a microwave peptide synthesizer (CEM Discover SPS).
   a. Calculate the amount of resin (Rink-amide MBHA resin) needed based on the substitution ratio of the resin and the synthesis scale. Swell the resin in dimethylformamide (DMF) in a reaction vessel (RV) for 15 min.
   b. Remove Fmoc-protecting group from the resin using a deprotection solution containing 20% Piperidine and 0.1 M HOBt in DMF. Use the parameters listed in the table below for microwave-assisted Fmoc-deprotection (for all Fmoc-amino acids):
<table>
<thead>
<tr>
<th>Synthesis scale</th>
<th>0.1 mmole</th>
<th>0.2 mmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power</td>
<td>20 W</td>
<td>50 W</td>
</tr>
<tr>
<td>Temperature</td>
<td>75 °C</td>
<td>75 °C</td>
</tr>
<tr>
<td>Time</td>
<td>3 min</td>
<td>3 min</td>
</tr>
</tbody>
</table>
   c. Following deprotection, rinse the resin with DMF and perform Ninhydrin test to confirm the removal of Fmoc (exposure of N-terminal amine). Resin should turn blue after 2 min at 95 °C.
   d. After successful deprotection, couple the first Fmoc-amino acid at the C-terminus (synthesis runs from C- to N-terminus) in an activator base solution (0.28 M Diisopropylethylamine (DIEA) in DMF) with the parameters listed in the table below.
<table>
<thead>
<tr>
<th>Synthesis scale</th>
<th>0.1 mmole</th>
<th>0.2 mmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power</td>
<td>20 W</td>
<td>50 W</td>
</tr>
<tr>
<td>Temperature*</td>
<td>75 °C</td>
<td>75 °C</td>
</tr>
<tr>
<td>Time*</td>
<td>5 min</td>
<td>5 min</td>
</tr>
</tbody>
</table>
   * For Cyst and His, use 50 °C and 10 min to reduce racemization.
   e. Rinse the resin and perform Ninhydrin test to qualitatively confirm the completion of coupling (disappearance of N-terminal amine). Resin should be clear after 5 min at 95 °C. Repeat coupling step (A.2.d) if Ninhydrin test returns positive result (blue color).
   f. Repeat deprotection (step A.2.b) and coupling (step A.2.d) for additional amino acids in the sequence ending with a final deprotection step.
   g. Drain the RV, rinse resin with 25 ml of dichloromethane (DCM). This step is to rinse off DMF, which is a base and will hinder peptide cleavage.
   h. Prepare cleavage cocktail by mixing 250 mg phenol in 4.75 ml Trifluoroacetic acid (TFA), 0.125 ml Triisopropylsilane (TIS) and 0.125 ml ddH₂O.
   i. Add cleavage solution into the RV and cleave for 30 min in the microwave peptide synthesizer (Power = 20 W; Temperature = 38 °C).
   j. Collect the peptide solution in a 50 ml centrifuge tube using vacuum manifold.
   k. Precipitate cleaved peptide in 40 ml of ether, vortex the tube and centrifuge at 3,000 rpm for 5 min to collect the peptide.
   l. Drain the supernatant and repeat step A.2.k twice.
   m. Collect and dry peptide in vacuum.
   n. Purify the peptide using HPLC and characterize it with mass spectrometry.

**B. Material Preparation and Sterilization**

1. Prepare 20 wt% PEG4NB solution in PBS, vortex the mixture until the macromer dissolves completely, and sterilize the macromer solution using a syringe filter. Store the sterilized solution at -20 °C (prepare aliquots for long-term storage).
2. Prepare and sterilize 2 wt% photoinitiator (LAP) solution in PBS. Store the sterilized solution at room temperature protected from light.
3. Dissolve the peptide (CGGYC) in PBS, vortex the mixture and syringe filter the solution for sterilization. Aliquot the peptide solution and store at -20 °C until use (prevent freeze-thaw cycles).
4. Determine thiol (-SH) concentration in the prepared peptide solution using Ellman's reagent (follow the manufacturer's protocol). This step will give accurate peptide concentration (i.e., [SH]/2) that is required for calculating the peptide amount to be used in thiol-ene photo-click reactions.
5. Prepare gelation molds by cutting the top portion off of sterile 1 ml disposable syringes using a razor blade (open tip for gel removal). Sterilize the syringe molds by autoclave.
6. Based on the desired gel formulation (thiol to ene ratio = 1) and concentrations of stock materials, calculate the required volume of polymer, peptide crosslinker, photoinitiator, and buffer solutions required.

C. Cell Preparation

1. Equilibrate cell culture medium (high glucose DMEM containing 10% fetal bovine serum; Antibiotic-Antimycotic with 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml Fungizone; and 50 μM β-mercaptoethanol), Hank's balanced salt solution (HBSS, Ca²⁺, Mg²⁺ free), and Trypsin-EDTA to 37 °C.

2. Remove flasks containing MIN6 β-cells from CO₂ incubator and place the flasks in the laminar flow hood.

3. Aspirate cell culture media from the flasks and rinse the cell with HBSS.

4. Trypsinize the cells using 3 ml of 2X (0.1%) Trypsin-EDTA and incubate the flasks for 4 min at 37 °C and 5% CO₂.
   • Note: Use 2X trypsin solution allows complete dissociation of MIN6 β-cells into single cell suspension.

5. After incubation, tap the flasks on the surface of the hood gently to completely detach the cells. Confirm the detachment and dissociation of the cells using a microscope.

6. Add equal volume of cell culture medium to neutralize the trypsin. Mix the solution well by gentle pipetting, then transfer the mixture to a canonical tube and centrifuge for 5 min at 1,000 rpm.

7. Aspirate the supernatant and gently re-suspend the cells in 4 - 5 ml of culture media.

8. Determine cell density using 50% Trypan blue buffer in a hemocytometer.

D. Hydrogel Fabrication and Cell Encapsulation

1. Once the cells are ready, mix (based on a 1:1 thiol to ene ratio) the required volumes of PEG4NB, CGGYC, LAP, cell solution, and HBSS in a microcentrifuge tube.

2. Mix the solution gently using a pipette to obtain a homogeneous mixture and add 25 μl of the resulting solution to a syringe mold. Expose the syringe to UV light (365 nm, 5 mW/cm²) for 2 min.

3. Following photopolymerization, plunge gels into a sterile 24-well plate containing cell culture medium and incubate the hydrogels in 37 °C and 5% CO₂.

4. Wash the cell-laden hydrogels in cell culture medium for 30 min to rinse off loosely attached cells and un-crosslinked macromers. Transfer the gels into fresh medium.

5. Refresh cell culture media every 2 to 3 days.

E. Cell Viability Assay

Encapsulated cell morphology can be observed using light microscope (since the synthesized hydrogels are transparent). Cell viability can be visualized and measured qualitatively using Live/Dead staining and quantitatively using AlamarBlue reagent.

E.1. Live/Dead staining

1. Thaw Live/Dead staining reagents at room temperature.

2. In a canonical tube add PBS according to the number of samples (n) to be stained:

   Total volume of PBS=n × 500 μl

3. Pipette 0.25 μl of Calcein AM and 2 μl of EthD-1 per 1 ml of PBS to the canonical tube containing PBS. Vortex the solution to mix the components.

4. Incubate cell-laden hydrogels in Live/Dead staining solution (0.5 ml/sample) for 1 hr at room temperature on an orbital shaker.

5. Using a pasteur pipette, remove the dye solution and rinse the sample twice with PBS.

6. Place sample on a cover slip or glass slide. Observe and image cells using a confocal microscope.

E.2. AlamarBlue Assay

1. Prepare 10 v/v% AlamarBlue solution in cell culture medium.

2. Remove hydrogels from incubator and aspirate the media out of each well without making any contact with the hydrogel.

3. Incubate cell-laden hydrogels and an empty well (negative control) in 500 μl of 10% AlamarBlue solution for 16 hr.

   • Higher cellular metabolic activity reduces the dye to give higher fluorescence reading.
   • Be sure to include at least two wells of 10% Alamarblue solution as blanks. When analyzing the data, subtract the averaged blank fluorescence value from the fluorescence reading of the samples.

F. Chymotrypsin Mediated Gel Erosion and Spheroid Recovery

1. Prepare 1 mg/ml of Trypsin-free α-chymotrypsin solution in HBBS and sterile filter the solution.

2. Incubate hydrogels in chymotrypsin solution (500 μl for each gel) at room temperature with gentle shaking until complete gel erosion is achieved.
   • For a 25 μl gel, the time for complete erosion is approximately 5 min.
3. Place the plate on ice for a few minutes to reduce the activity of chymotrypsin.
4. Transfer the cell solution in 1 ml tube and centrifuge at 300 rpm, 4 °C for 2 min.
5. Carefully remove the supernatant using a pasture pipette and gently resuspend the spheroids in HBSS.
6. Transfer the recovered spheroid solution to a 24-well plate and place the plate on ice to allow the spheroids to settle down.
7. For size analysis, acquire phase contrast images of the settled spheroids using a light microscope. Measure recovered spheroid diameters using software such as Nikon Element software or ImageJ.

G. Functional Assay of Recovered Spheroids

G.1. Glucose stimulated insulin secretion from the recovered cell spheroids

1. In a 24 well plate, incubate the gels in 500 μl of 2 mM low glucose Kerbs-Ringer buffer (KRB) for 1 hr.
2. Erode the gel and recover cell spheroids using steps F.1 to F.6.
3. Aspirate the supernatant carefully without making any contact with the cell spheroids and resuspend the spheroids in 500 μl of 2 mM low glucose KRB.
4. Split the remaining cell spheroid solution into two microcentrifuge tubes and centrifuge for 2 min at 300 rpm and 4 °C.
5. Aspirate supernatant (almost to the bottom) and resuspend the first half of cell spheroids in 1 ml of 25 mM high glucose KRB and second half in 1 ml of 2 mM low glucose KRB.
6. Gently pipette and transfer the solution containing recovered cell spheroids to a 24-well plate. Incubate the plate for 1 hr at 37 °C and 5% CO₂.
7. Following incubation, transfer the solution to a microcentrifuge tube and centrifuge for 2 min at 1,000 rpm and 4 °C.
8. Transfer 500 μl of the supernatant from the centrifuged tube to another microcentrifuge tube and store at 4 °C for insulin ELISA.
9. Perform insulin ELISA following manufacturer's protocol.

G.2. CellTiter Glo Assay

1. ATP standards: Prepare a 10 μM ATP solution and perform serial dilutions to obtain a series of 8 standard solutions.
2. To a white 96 well plate, add 50 μl of cell spheroid solution (from step G.1.6) and standards.
3. To each well containing samples and standards, add 50 μl of CellTiter Glo reagent and incubate at room temperature for 15 min.
4. Following incubation, measure luminescence count of the samples and standards using a microplate reader and calibrate sample ATP concentrations using a linear regression curve generated by the ATP standards.
5. Determine the total ATP concentration in sample and multiply by the sample dilution factor to get the intracellular ATP concentration in one cell-laden hydrogel.

G.3. Determine insulin secretion

1. Calculate the amount of insulin secreted from the recovered spheroids by taking the ratio of the amount of insulin secreted in high glucose medium to amount of insulin secreted in low glucose medium.
2. Normalize the quantified insulin content of cell spheroids with respective amount of intracellular ATP of the recovered cell spheroids.

Representative Results

Figures 1-4 show representative results for encapsulation, survival, proliferation, spheroid formation, and spheroid recovery in thiol-ene hydrogels. Figure 1 shows the reaction schematic of (1) step-growth thiol-ene photopolymerization using PEG4NB and CGGYC, and (2) chymotrypsin mediated gel erosion which follows a surface erosion mechanism. Figures 2 and 3 present viability results obtained using Live/Dead staining and AlamarBlue assay. We observe that cells proliferated in PEG4NB-CGGYC hydrogels even at low cell packing density, indicating the cytocompatibility of thiol-ene hydrogel system. Figure 4 shows phase contrast images of encapsulated and recovered β-cell spheroids, as well as the size distribution of the recovered β-cell spheroids.
Figure 1. Schematic of step-growth thiol-ene photo-click reaction using PEG4NB and CGGYC to form PEG-peptide conjugate. Hydrogels can be eroded rapidly by chymotrypsin treatment.
Figure 2. Initial viability and cell spheroid formed in PEG4NB/CGGYC hydrogels. (a) Live/Dead staining was performed immediately following photo-encapsulation. (b) Live/Dead staining performed after 10 days of in vitro culture. Representative confocal z-stack images of MIN6 β-cells encapsulated in 4wt% PEG4NB/CGGYC hydrogels (2×10^6 cells/ml, scale = 100 μm). Cell viability was defined as the percentage of live (green) cells over total cell (green + red) count. (2×10^6 cells/ml, N=4, mean ± SD).

Figure 3. Metabolic activity of MIN6 β-cells measured as a function of time by AlamarBlue reagent (N=3, mean ± SD). MIN6 β-cells encapsulated in 3wt%, 4wt% and 5wt% PEG4NB/CGGYC hydrogels at 2×10^6 cells/ml.
Figure 4. Characterization of recovered MIN6 β-cell spheroids. Cell spheroids were recovered from PEG4NB/CGGYC using 40 μM chymotrypsin after 10 days of in vitro culture. (a) Representative phase contrast image of encapsulated β-cell spheroids. (b) Representative phase contrast image of recovered β-cell spheroids. (c) Distribution of spheroid diameters (Cell density = 1×10^7 cells/ml).

Discussion

The described protocol presents details on easy encapsulation of cells in thiol-ene hydrogels formed by step-growth photopolymerization. While a stoichiometric ratio of 1:1 of norbornene to thiol functional groups was used in this protocol, the ratio can be adjusted depending on the experiments. In addition to a correct formulation, it is important to maintain homogeneity in the pre-polymer solution. In particular, use gentle pipetting to ensure that cells are well distributed in the pre-polymer solution in order to avoid clumping of cell and variation in gel properties. Although a polymerization time of 2 min was used for gel crosslinking, the gel point for thiol-ene hydrogels is less than 10 sec in most cell encapsulation-relevant formulations (e.g., gel point for 4 wt% PEG4NB/CGGYC hydrogel is 7 ± 2 sec and 5 wt% is 6 ± 1 sec). The rapid gelation of this hydrogel system swiftly locks the cells in place, resulting in better cell distribution in 3D compared to other photopolymerization schemes that take minutes or even hours to reach gel point.\(^7,20\)

Additionally, β-cell spheroids naturally formed in thiol-ene hydrogels were recovered by chymotrypsin-mediated gel erosion. Chymotrypsin, however, is an enzyme in the trypsin family and high concentration or long incubation time of this enzyme may negatively affect cell-cell interactions or even disrupt the spheroid architecture during recovery. In order to avoid this potential limitation, hydrogels may be crosslinked by other thiolated substrates and gel erosion can still be achieved by suitable enzymes that do not cause adverse cell-cell interaction.

Overall, thiol-ene hydrogels provide a cytocompatible environment for promoting the proliferation of β-cells in 3D. This system may be used to culture and study the physiological behavior of various cell types in 3D. Furthermore, this system allows for facile recovery of cell constructs formed within the hydrogel matrix for further functional and biological characterizations. This diverse and cytocompatible hydrogel system provides gel platform for engineering complex 3D tissues for regenerative medicine application.
Disclosures

No conflicts of interest declared.

Acknowledgements

This project was funded by NIH (R21EB013717) and IUPUI OVCR (RSFG). The author thanks Ms. Han Shih for her technical assistance.

References