

Video Article

Tracking Hypoxic Signaling within Encapsulated Cell Aggregates

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DOI: 10.3791/3521

Keywords: Bioengineering, Issue 58, Cell encapsulation, PEG, cell aggregation, hypoxia, insulin secretion, fluorescent imaging,

Date Published: 12/16/2011

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Citation: Skiles, M.L., Sahai, S., Blanchette, J.O. Tracking Hypoxic Signaling within Encapsulated Cell Aggregates. *J. Vis. Exp.* (58), e3521, DOI : 10.3791/3521 (2011).

Abstract

In Diabetes mellitus type 1, autoimmune destruction of the pancreatic β -cells results in loss of insulin production and potentially lethal hyperglycemia. As an alternative treatment option to exogenous insulin injection, transplantation of functional pancreatic tissue has been explored^{1,2}. This approach offers the promise of a more natural, long-term restoration of normoglycemia. Protection of the donor tissue from the host's immune system is required to prevent rejection and encapsulation is a method used to help achieve this aim.

Biologically-derived materials, such as alginate³ and agarose⁴, have been the traditional choice for capsule construction but may induce inflammation or fibrotic overgrowth⁵ which can impede nutrient and oxygen transport. Alternatively, synthetic poly(ethylene glycol) (PEG)-based hydrogels are non-degrading, easily functionalized, available at high purity, have controllable pore size, and are extremely biocompatible^{6,7,8}. As an additional benefit, PEG hydrogels may be formed rapidly in a simple photo-crosslinking reaction that does not require application of non-physiological temperatures^{6,7}. Such a procedure is described here. In the crosslinking reaction, UV degradation of the photoinitiator, 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure 2959), produces free radicals which attack the vinyl carbon-carbon double bonds of dimethacrylated PEG (PEGDM) inducing crosslinking at the chain ends. Crosslinking can be achieved within 10 minutes. PEG hydrogels constructed in such a manner have been shown to favorably support cells^{7,9}, and the low photoinitiator concentration and brief exposure to UV irradiation is not detrimental to viability and function of the encapsulated tissue¹⁰. While we methacrylate our PEG with the method described below, PEGDM can also be directly purchased from vendors such as Sigma.

An inherent consequence of encapsulation is isolation of the cells from a vascular network. Supply of nutrients, notably oxygen, is therefore reduced and limited by diffusion. This reduced oxygen availability may especially impact β -cells whose insulin secretory function is highly dependent on oxygen¹¹⁻¹³. Capsule composition and geometry will also impact diffusion rates and lengths for oxygen. Therefore, we also describe a technique for identifying hypoxic cells within our PEG capsules. Infection of the cells with a recombinant adenovirus allows for a fluorescent signal to be produced when intracellular hypoxia-inducible factor (HIF) pathways are activated¹⁴. As HIFs are the primary regulators of the transcriptional response to hypoxia, they represent an ideal target marker for detection of hypoxic signaling¹⁵. This approach allows for easy and rapid detection of hypoxic cells. Briefly, the adenovirus has the sequence for a red fluorescent protein (Ds Red DR from Clontech) under the control of a hypoxia-responsive element (HRE) trimer. Stabilization of HIF-1 by low oxygen conditions will drive transcription of the fluorescent protein (Figure 1). Additional details on the construction of this virus have been published previously¹⁵. The virus is stored in 10% glycerol at -80°C as many 150 μ L aliquots in 1.5 mL centrifuge tubes at a concentration of 3.4×10^{10} pfu/mL.

Previous studies in our lab have shown that MIN6 cells encapsulated as aggregates maintain their viability throughout 4 weeks of culture in 20% oxygen. MIN6 aggregates cultured at 2 or 1% oxygen showed both signs of necrotic cells (still about 85-90% viable) by staining with ethidium bromide as well as morphological changes relative to cells in 20% oxygen. The smooth spherical shape of the aggregates displayed at 20% was lost and aggregates appeared more like disorganized groups of cells. While the low oxygen stress does not cause a pronounced drop in viability, it is clearly impacting MIN6 aggregation and function as measured by glucose-stimulated insulin secretion¹⁵. Western blot analysis of encapsulated cells in 20% and 1% oxygen also showed a significant increase in HIF-1 α for cells cultured in the low oxygen conditions which correlates with the expression of the DsRed DR protein.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3521/>

Protocol

1. PEGDM synthesis and photoactive PEGDM macromer solution preparation

1. Weigh 2g of PEG (linear, 10,000Da) into a 40mL glass vial with a hard plastic cap.
2. Add approximately 308 μ L of methacrylic anhydride and loosely cap the vial.
3. Microwave the vial on high for 2 minutes in a standard domestic microwave. Wearing heat resistant gloves, thoroughly vortex the vial, then microwave on high for an additional 5 minutes.

4. Briefly allow the vial to cool enough to be handled with gloves. Uncap it and slowly add methylene chloride while swirling the vial. Add enough so that the solution appears clear and homogeneous, vortexing the sample as needed. The total volume of methylene chloride used should be 1.5 - 2 mL.
5. Precipitate PEGDM in 200 mL of cold, stirred diethyl ether by adding the solution dropwise.
6. Collect the PEGDM by vacuum filtration and allow it to dry.
7. Dissolve the PEGDM in an adequate amount of deionized water (typically 100 -150 mL).
8. Dialyze the solution against deionized water for 5 days in dialysis tubing with a molecular weight cut-off of 1,000 Da. Replace the water once daily.
9. Aliquot the solution into appropriate containers and freeze at -80°C overnight. Lyophilize the solution for 4 days to yield a purified white PEGDM powder. Store the powder at 4°C when not in use.
10. To prepare a photoactive PEGDM solution, first prepare a 0.5 weight percent photoinitiator stock solution by dissolving Irgacure 2959 in deionized water. Vortex the solution and store it in the dark.
11. Prepare a 10 wt% PEGDM and 0.025 wt% Irgacure 2959 solution in Hank's Balanced Salt Solution (HBSS). Vortex thoroughly to ensure dissolution of the PEGDM. We typically prepare 1mL of this solution at a time.
12. Sterilize the solution by filtering it through a 0.2 µm syringe filter into a 1.8 mL microcentrifuge tube. Wrap the tube in aluminum foil and store at 4°C

2. Culture, infection, and aggregation of MIN6 cells

1. MIN6 cells are cultured in RPMI 1640 medium supplemented with 10% FBS, 7mM glucose, 100 units/mL penicillin/streptomycin, and 0.5 µg/mL amphotericin B in a humidified environment at 37°C and 5% CO₂.
2. To release the cells from the treated culture flask surface, aspirate the medium, rinse the cells with 37°C calcium/magnesium-free HBSS, aspirate the HBSS, then add 2 mL of 37°C trypsin-EDTA solution and allow the cells to incubate for 3 minutes.
3. Firmly jar the side of the flask to knock the cells free, then add 8 mL of 37°C medium and vigorously pipette the cell suspension up and down taking care not to introduce bubbles.
4. Pipette the cells into a sterile 15 mL centrifuge tube and perform a cell count using a hemocytometer or other cell counting procedure.
5. In preparing to infect the cells with the maker virus, first determine the total number of cells to be infected and calculate the volume of virus suspension required to achieve the desired multiplicity of infection (MOI) (50 to 100 infectious particles per cell).
6. Dilute the virus in HBSS by carefully pipeting the proper amount of virus suspension into a pre-aliquoted volume of HBSS in a 1.8 mL microcentrifuge tube. 50 µL of HBSS per well of aggregating cells is appropriate.
7. Disperse the cells by pipeting them up and down in their tube, then pipet the necessary volume to achieve 400,000 cells per well into the wells of a 6-well suspension culture plate.
8. Add the appropriate volume of diluted virus to each well to achieve the desired MOI.
9. Add medium to each well to bring the total volume up to 1.7 mL.
10. Place the plate on an orbital shaker inside the incubator. Set the shaker on a low setting so that the medium gently washes around the wells (~100 rpm). Allow the cells to aggregate for 24-36 hours. Aggregates with an approximate diameter of 75-175 µm should be formed.

3. Encapsulation of cells in PEGDM

1. Cells may be encapsulated as a dispersion (step 2.4) or following aggregation (step 2.10). Since the hydrogel precursor PEGDM solution is a liquid, cells may tend to settle prior to crosslinking and hydrogel formation. If settling is expected to occur rapidly, such as with larger aggregates, it may be beneficial to produce the hydrogel in two steps so that the cells settle to a central plane of the gel. A one-step hydrogel synthesis procedure appropriate for dispersed cells is shown (**Figure 2**) and the two-step procedure is shown as well (**Figure 3**) and detailed below (3.2-3.9).
2. Create a vessel in which the hydrogel will be formed by cutting the tapered tip off of a 1 mL plastic syringe and placing it open end up in a rack.
3. Pipet 20 µL of photoactive PEGDM solution into the open end of the syringe onto the plunger making sure the volume covers the entire plunger surface. Adjust the plunger in small increments to achieve a flat fluid surface.
4. Place the pipet in the rack and position the rack underneath a UV lamp (365nm, ~7 mW/cm²) for approximately 8 minutes to allow for hydrogel crosslinking. During this time, preparation of the cells may be begun
5. Pipet a volume containing the desired number of cells/aggregates into a 1.8 mL microcentrifuge tube, cap, and centrifuge the tube at 130 g for 5 minutes.
6. Using a micropipettor, carefully remove the media without disturbing the cell pellet at the tip of the tube. As the media head approaches the pellet, it may be helpful to use a finer, 10 µL pipet tip.
7. Gently resuspend the cell pellet in 20 µL of photoactive PEGDM solution (use a wide-mouthed pipet tip if working with aggregates) and add the cell suspension to the syringe on top of the previously crosslinked hydrogel portion.
8. Expose the syringe to an additional 8 minutes of UV light to complete construction of the hydrogel. When finished, the cells should be fully encapsulated within the cylindrical hydrogel (**Figure 3**).
9. Eject the hydrogel from the syringe and into 1 mL of HBSS in the well of a 24-well plate to briefly wash the gel. Transfer the gel to 1 mL of media in the well of a 24-well plate and culture under the desired conditions.

4. Hypoxia tracking

1. At any point during the culture, image the cells using fluorescent microscopy to track the initiation of hypoxic signaling. Depending on what is being imaged you can image in the multi-well plate or transfer the gel to a microscope slide prior to placement on the microscope stage. Peak excitation of Ds Red is achieved at 556nm and peak emission occurs at 586nm. Emission is fairly intense and thus photobleaching has not been observed to be problematic. Lag time between initiation of protein production and first signal detection is approximately 12 hours. Signal is specific enough to identify individual signaling cells, but resolution may be inadequate for determining intracellular signal localization. In signaling cells, signal is typically observed uniformly throughout the cytoplasm. Signal intensity can also increase with extended exposure to hypoxia, though we have not yet fully determined if this is due to increased protein expression, overall protein accumulation, or delayed protein maturation.

5. Representative results

A representative example of MIN6 aggregates encapsulated in a PEG hydrogel is shown in **Figure 4**. The crosslinked gel will be solid throughout, taking the shape of the vessel in which the reaction was performed. A gel with smooth outer surfaces is preferable for implantation to aid in prevention of a foreign body response. Within the gel, cell aggregates should be fully enclosed in the matrix and homogenously distributed to allow for better nutrient transport.

Representative images of hypoxic signaling in MIN6 aggregates are pictured in **Figure 5**. Identical MIN6 aggregates infected with the marker virus were cultured in either 20% O₂ (5a) or 1% O₂ (5b) for 44 hours before image capture.



Figure 1. Schematic of the activity of our hypoxia marker system. Adenoviral insertion of the Ds Red DR gene and upstream HRE promoter allows for hypoxia-induced production of the fluorescent protein under control of HIF-1.

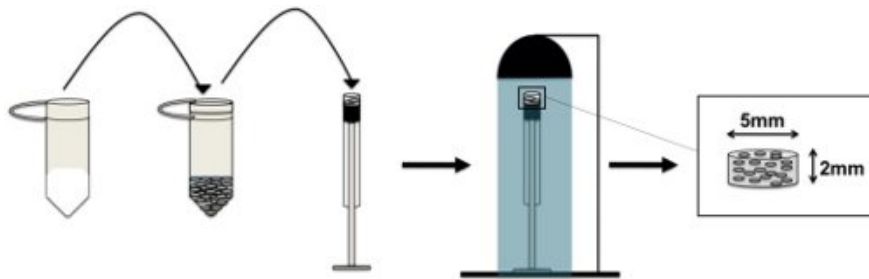


Figure 2. Procedural flow chart for the single-step encapsulation of dispersed MIN6 cells in a crosslinked PEGDM hydrogel. For each gel, the dispersed cells are suspended in 40µL of the photoactive PEGDM macromer solution. This is placed in a decapitated 1mL syringe and the hydrogel is formed under 365nm UV light after 10-12 minutes. Upon completion, the hydrogel disk is removed from the syringe, washed and placed in a medium-filled well plate for incubation.

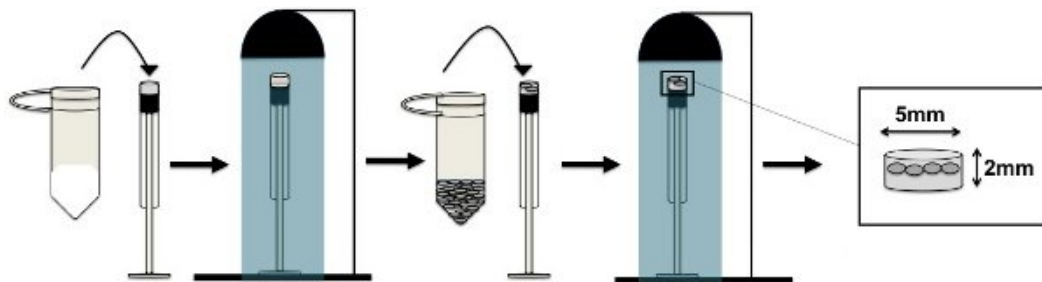


Figure 3. Procedural flow chart for the dual-step encapsulation of aggregated MIN6 cells in a crosslinked PEGDM hydrogel. For each gel, a half-gel is first formed by UV crosslinking of 20µL of the photoactive PEGDM macromer solution for 8 minutes. MIN6 aggregates are carefully suspended in an additional 20µL of photoactive macromer solution which is added on top of the pre-formed half-gel. The full gel is formed by an additional 8 minutes of UV exposure with aggregates fully encapsulated in the medial plane of the gel.

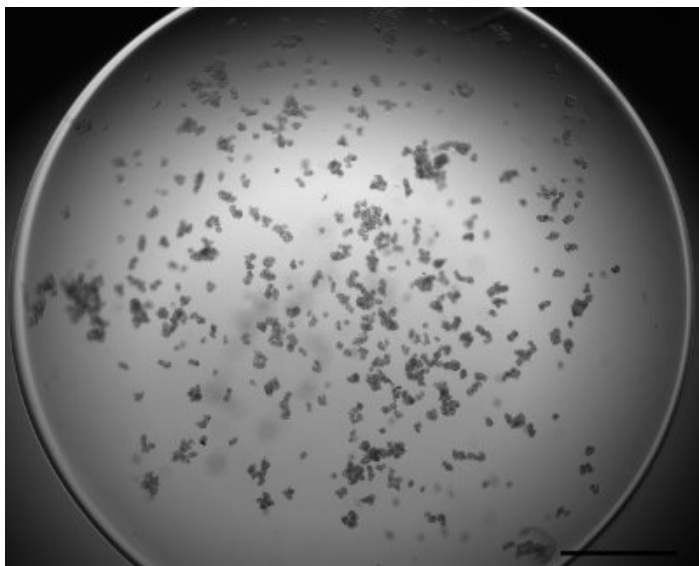


Figure 4. Image of a 40 μ L hydrogel under 20X magnification. MIN6 aggregates (~400,000 total cells) are clearly seen within the gel. Hydrogel diameter is approximately 6mm (bar = 1mm).

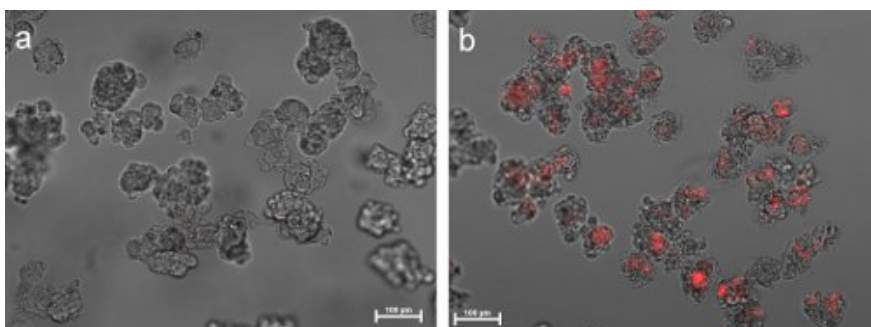


Figure 5. Fluorescent hypoxia signaling in aggregated MIN6 cells in a PEGDM hydrogel. Cells that were encapsulated and then placed in incubation at 20% O₂ for 44 hours do not display hypoxia signaling (a) while cells that were encapsulated then incubated in 2% O₂ for 44 hours display clear, ubiquitous signal. (bar = 100 μ m) (b).

Discussion

The method presented here offers a quick and simple technique for cell encapsulation in a PEG hydrogel with minimal use of non-physiological conditions. PEG represents a very useful encapsulation material for its biocompatibility and ease of modification. Simple variation of PEG percentage in the photoactive solution, for instance, may be used to adjust mechanical properties, such as compressive modulus, and transport properties through pore size. Also, PEG is easily modified by the addition of side chains. PEG hydrogels, therefore, represent both a promising clinical device and a flexible platform for *in vitro* research

A method for tracking hypoxia in PEG-encapsulated cells has also been presented. This method is useful for the simplicity of hypoxia detection and for avoiding the need to sacrifice the cells of interest. The technique may be applied to a variety of types of cells in a variety of conditions making its usefulness broad. For instance, hypoxia as a cue for stem cell differentiation may be tracked in stem cell micromass cultures. However, this method can only be applied to disperse cell systems or system in which dispersed cells are later aggregated. Also, detection of the fluorescent signal may be difficult in larger or denser tissues.

Disclosures

No conflicts of interest declared.

Acknowledgements

Thanks to the Kristi Anseth lab of the University of Colorado, Boulder for generously supplying MIN6 cells. Funding for this project has been provided by the NSF.

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