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Methods for Incorporating Oxygen Generating Biomaterials into Cell Culture and Microcapsule Systems

John Patrick McQuilling^{1,2}, Emmanuel C Opara^{1,2,+}

¹Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

²Virginia Tech-Wake Forest University School of Biomedical Engineering and Sciences, Winston-Salem, NC 27157, USA

Summary/Abstract

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A major obstacle to long-term performance of tissue construct implants in regenerative medicine is the inherent hypoxia to which cells in the engineered construct are exposed prior to vascularization of the implant. Various approaches are currently being designed to address this problem. An emerging area of interest on this issue is the use of peroxide-based materials to generate oxygen during the critical period of extended hypoxia that occurs from the time cells are in culture waiting to be used in tissue engineering through the immediate post- implant period. In this chapter we provide protocols that we have developed for using these chemical oxygen generators in cell culture and tissue constructs as illustrated by pancreatic islet cell microencapsulation.

Keywords

Oxygen Generating Biomaterials; Calcium Peroxide; Sodium Percarbonate; Alginate; Microencapsulation

1. Introduction

One hurdle for many cell and tissue based therapies is overcoming hypoxia during the immediate post-transplant period prior to the onset of angiogenesis.(1,2) This is particularly challenging with cells or tissue with exceptionally high metabolic demands such as skeletal muscle or pancreatic islets.(3,4) There are several approaches to reducing hypoxic injury following implantation including the pre-vascularization of the transplantation site (5) reducing the time to engraftment with the localized delivery of angiogenic growth factors (6,7) the use or incorporation of oxygen carriers within biomaterial constructs (8) and locally supplying oxygen with oxygen generating materials.

Compared to pre-vascularization strategies, oxygen generating materials have the advantage of requiring only a single surgery. In contrast to strategies which utilize a localized delivery

⁺Address for correspondence: Dr. Emmanuel C Opara, Institute for Regenerative Medicine, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157, USA, Tel: 336-713-1297, Fax: 336-713-7290, eopara@wakehealth.edu.

of angiogenic growth factors to decrease the time to engraftment, oxygen generating materials immediately provide implanted cells or tissues with oxygen. Unlike oxygen carrying strategies such as hemoglobin-based materials, oxygen generating materials do not require a vasculature supply or oxygen reservoir. Oxygen generating materials include calcium peroxide (CPO) and sodium percarbonate (SPO) which have both been applied to several tissue engineering applications.

Sodium percarbonate (SPO) is an adduct of sodium carbonate and hydrogen peroxide which in the presence of water will rapidly decompose to generate oxygen as shown in equations 1 and 2 below:

SPO particles, similar to those illustrated in Figure 1 A, have previously been utilized within a poly(d,l-lactide-co-glycolide) (PGLA) scaffold to reduce necrosis and cellular apoptosis in an ischemic flap model and has also been administered via intramuscular injection to an ischemic muscle injury which resulted in improved functional outcomes.(9,10)

CPO decomposes in the presence of water to form H_2O_2 which then decomposes to oxygen and water, as shown in equations 3 and 4.

CPO particles, illustrated in Figure 1B, which decompose at a slower rate than SPO, have been utilized within a PLGA scaffold and shown to improve fibroblast proliferation over a 10 day period in hypoxic conditions.(11) When incorporated into a silicone construct, CPO was able to generate oxygen for up to a six week period. Using this system for pancreatic islets culture a significant reduction in hypoxia-induced cell death and dysfunction was observed for up to 48 hours. It has also been shown to improve MIN6 cell survival for up to three weeks.(12)

While there is growing evidence that oxygen generating materials can be utilized to prevent hypoxic damage to cells and tissues, this is a novel technology which is still under development. One challenge to implementing this technology is controlling the rate of oxygen generation so that there is enough oxygen to prevent hypoxia, but not too much that the angiogenic process is disrupted.(13) Additionally these materials often have a burst release, which results in the buildup of hydrogen peroxide that is cytotoxic, and this buildup in turn increases the chance of hydroxyl radical formation with further damage to tissues. (14) There are several factors which play a role in controlling the duration and rate of oxygen release including the local pH (14), particle size, the purity of either the SPO or

CPO, the applications of hydrophobic coatings such as steric acid or paraffin, and the materials used to encapsulate the oxygen generating material. Additionally, the use of antioxidants, in particular, catalase is recommended to mitigate cytotoxicity caused by oxidative stress from these materials.

Here we present two different methods for utilizing these two oxygen generating materials. The first method outlines the process for producing a culture system designed to generate oxygen from sodium percarbonate encapsulated within a layer of polydimethylsiloxane (PDMS). While SPO typically reacts rapidly in the presence of water and under physiological conditions and catalase can completely degrade within minutes, the addition of the hydrophobic PDMS greatly slows this reaction enabling oxygen generation to occur over a period of days as water slowly comes into contact with the SPO between the layers of PDMS. The second method outlines the process for fabricating alginate microspheres with CPO to generate oxygen to encapsulated islets. In order to prevent cell death as a result of the initial burst release of CPO, CPO was pre-incubated with PBS overnight and then washed with PBS to remove smaller particles. Both systems have been tested with pancreatic islets under relatively hypoxic conditions and have been shown to reduce damage from oxidative stress; however it is recommended that the concentration and particle size of these oxygen generating materials as well as the antioxidant loads be experimentally determined for each cell or tissue type tested with these systems.

2. Materials

2.1. Materials for Oxygen Generating Culture System

- 2.1.1. Sodium Percarbonate (SPO) (Sigma-Aldrich)
- 2.1.2. Sylgard 184 Silicone elastomer kit (World Precision Instruments, Sarasota FL)
- 2.1.3. 150 mm x 20 mm plastic Petri dish
- 2.1.4. 90 mg Sodium Percarbonate particles ~1 mm in diameter (Sigma-Aldrich)
- 2.1.5. Bovine Catalase (Sigma-Aldrich)
- 2.1.6. Vacuum Oven

2.2. Materials for Oxygen Generating Microcapsules

- 2.2.1. Calcium Peroxide (Sigma-Aldrich)
- 2.2.2. Phosphate Buffered Saline (without Ca^{2+} or Mg^{2+})
- 2.2.3. 1.5% Alginate (Pronova UP LVM and UP LVG, Novamatrix, Sandvika Norway)
- 2.2.4. 0.1% Poly-L- Ornithine (PLO) (P5061, Sigma-Aldrich)
- 2.2.5. 100 mM CaCl_2 solution (C614–10, Fischer Scientific, Waltham, MA, USA)
- 2.2.6. 55 mM Sodium citrate solution (S467–3, Fischer Scientific)

2.2.7. 0.9% Sodium chloride solution (normal saline) (71376–5KG, Sigma-Aldrich)

2.2.8. 10 mM HEPES solution (H3375–2KG, Sigma-Aldrich)

2.2.9. Eight channel microfluidic encapsulation device (Department of Mechanical Engineering, Clemson University, SC - **See Note 1**)

3. Methods

3.1. Oxygen Generating Culture System

3.1.1. Following the manufacturer's instructions, prepare a 1:10 ratio (by weight) of Catalyst to PDMS

3.1.2. Add enough of the PDMS solution to form a 1 mm thick layer on the bottom of the petri dish

3.1.3. Remove air bubbles in PBMS by placing in a vacuum for 1 hour

3.1.4. Allow to cure at room temperature for 48 hours (**See Note 2**)

3.1.5. Evenly distribute SPO particles across the cured layer of PDMS

3.1.6. Prepare a second 1:10 mixture of catalyst and PDMS and evenly apply over SPO and bottom layer of silicone to form a top layer of silicone approximately 1 mm thick.

3.1.7. Remove air bubbles via vacuum for one hour at room temp

3.1.8. Allow 48 for final layer of silicone to cure

3.1.9. Sterilize via ethylene oxide or gamma irradiation prior to use. (**See Note 3**).

3.1.10. We recommend supplementing standard culture media with 100 U/mL of Catalase prior to use.

3.2. Oxygen Generating Microcapsules

3.2.1. Add 10 mg of CPO to 50 mL of sterile PBS and incubate over night at 37°C.

3.2.2. Allow CPO to settle by gravity to the bottom of the conical tube and aspirate PBS.

3.2.3. Wash with 50 mL PBS three times and allow particles to settle by gravity prior to aspirating.

3.2.4. Suspend remaining CPO particles in 1 mL alginate. (**See Note 4**)

3.2.5. Suspend islets in sodium alginate/CPO suspension, 1. Islets are suspended in a solution of sodium alginate (usually from 1.2 – 1.8% w/v made up in normal saline with 5,000 islets suspended in 1 mL of alginate)

3.2.6. Microspheres of alginate containing one or two islets (depending upon the alginate-islet ratio in the suspension) illustrated in Figure 2, are generated and allowed to gel into microbeads in a bath of 100 mM CaCl₂ dissolved in 10 mM HEPES solution at 4° C, pH 7.4 for 5–10 minutes.

3.2.7. Collect microspheres into a 50 mL conical tube and allow the capsules to settle with gravity.

3.2.8. Aspirate calcium chloride solution and wash three times in normal saline.

3.2.9. Incubate microspheres in 0.1% PLO for 30 minutes on ice.

3.2.10. Liquefaction of the alginate core of the microcapsules is achieved by a brief (2 minutes) incubation in 55 mM sodium citrate solution

3.2.11. Wash with normal saline three times as previously described.

3.2.12. Coat outer layer of capsule with 1.25% LVM for 5 minutes

3.2.13. Separate excess alginate from microcapsules and crosslink outer layer

3.2.14. Place microcapsules in culture using standard culture media supplemented with 100 U/mL Catalase.

4. Notes

4.1. The microfluidic device used here is constructed with in house materials as previously described. (15) This eight nozzle device uses standard syringe needles (gauges 20–27) and generates capsules at air jacket pressures at 2.5–10 psi and with alginate input flow rates of 0.5–2.5 mL/min.

4.2. SPO is temperature sensitive; curing the PDMS at high temperatures may result in the early degradation of SPO particles.

4.3. This system was originally designed to culture islets and since this is a suspension culture we have not included instructions for treating the cell culture surface for attachment cultures prior to sterilization.

4.4. The addition of CPO will begin to crosslink the alginate immediately after mixing. While pre-washing the particles with PBS will slow this process, encapsulation should occur quickly after preparing the alginate/CPO suspension.

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Figure 1:
SEM images of oxygen generating materials. (A) Representative image of SPO particles with an average diameter of $330 \pm 8 \mu\text{m}$. (B) Representative image of CPO particles with an average diameter of $2.78 \pm 1.83 \mu\text{m}$.

Figure 2:
(A) Phase contrast image of microencapsulated islets in alginate microcapsules with poly-L-orthinine semipermeable coatings (control). (B) Phase contrast images of islets co-encapsulated with CPO.