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COMMUNICATION

Injectable Oxygen-Generating Nanocomposite Hydrogels with Prolonged Oxygen Delivery for Enhanced Cell Proliferation under Hypoxic and Normoxic Conditions

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Abstract: We describe a new organic peroxide-based injectable biomaterial that was prepared by using Benzoylperoxide and Laponite incorporated alginate hydrogel (BPO-AlgL). BPO-AlgL show sustained release of O\textsubscript{2} over a period of 14 days and reduces the hypoxia-induced cell death. BPO-AlgL also promotes enhanced cell viability by providing sustained O\textsubscript{2} within the 3D AlgL scaffold. In addition, BPO-AlgL increases the O\textsubscript{2} level in the environment of the cells that led a decrease in the proliferation of malignant cells while that resulted in an increase in the viability of healthy fibroblast cells. Therefore, our new oxygen generating organic peroxide-based injectable 3D biomaterials have potential to contribute in the development of the next generation advanced tissue engineering biomaterials.

1. INTRODUCTION

Tissue engineering, an interdisciplinary field, intends to generate new tissue constructs to repair, improve or replace damaged tissues and organs. In the last years, much tissue engineering research has aimed at designing biomaterial scaffolds with the appropriate bio-functional and mechanical properties that can mimic the natural extracellular matrix (ECM) and thus support cell proliferation and guide cell differentiation in forming tissue structures.\textsuperscript{1}

Even though significant progress has been made on improving biomaterials for tissue engineering, some limitations remain, specifically in transplantation, healing large wounds and regenerating large organs. The main problems are that the engineered biomaterials are unable to be vascularized after implantation and that cells within 3D engineered constructs often cannot receive sufficient oxygen and nutrients in the initial phase after transplantation.\textsuperscript{2,4}

Oxygen (O\textsubscript{2}) is the most important nutrient necessary for cell survival. Insufficient oxygen delivery prevents cell migration and neovascularization and also reduces cell growth and differentiation. Furthermore, under insufficient oxygen conditions within the tissue-engineered construct, hypoxia often occurs, which reduces the efficiency of engineered tissues. Furthermore, under hypoxia malignant cells can resist antitumor drugs and increase their migratory and metastatic behaviour.\textsuperscript{5,6}

Researchers have considered delivering O\textsubscript{2} to engineered tissues. However, oxygen delivery to cells generally occurs as a burst release, and if oxygen is delivered as H\textsubscript{2}O\textsubscript{2}, it can damage cells and led to a decrease in cell proliferation and viability.\textsuperscript{7} This drawback remains a challenge and limits the development of suitable scaffolds that can supply sufficient oxygen to cells.

To overcome these problems, many approaches have been developed to generate oxygen-releasing biomaterials such that the release kinetics of oxygen can be controlled to occur over prolonged time periods, allowing for hypoxia to be avoided and for cell viability to be maintained before the engineered tissue is vascularized (1–2 weeks)\textsuperscript{8} by the host system.\textsuperscript{9,11} Various forms of these biomaterials have been generated by encapsulating O\textsubscript{2}-releasing molecules into polymer microspheres, adsorbing O\textsubscript{2}-carrying reagents into films, fibres, and scaffolds or using a dissolved form of O\textsubscript{2}-releasing molecules in medium. In such O\textsubscript{2}-generating biomaterials, the oxygen supply has included solid inorganic peroxides [e.g., CaO\textsubscript{2}, sodium percarbonate (\((\text{Na}_2\text{CO}_3)_2\cdot1.5\text{H}_2\text{O})\) and MgO\textsubscript{2}], liquid \(\text{H}_2\text{O}_2\), and perfluorocarbons.\textsuperscript{12–15} In addition, the vascularization of the engineered tissues has been aided by the use of certain 3D printing techniques and microfabrication methods.\textsuperscript{16–19}

These biomaterials have been utilized in tissue engineering applications for cardiac, skin, bone, muscle, and pancreas tissues.\textsuperscript{9,19–25} For example, Alemdar et al. reported calcium peroxide (CPO) embedded in gelatin methacryloyl (GelMA) as an O\textsubscript{2}-generating hydrogel. In this study using cardiac cells under hypoxic conditions, the CPO-GelMA hydrogels provided enough O\textsubscript{2} over a 5-day period to promote cardiac cell viability and reduce cell death.\textsuperscript{26} Fan et al. described an injectable
poly(lactide-co-glycolic acid) and poly(N-vinylpyrrolidone)/H₂O₂-based O₂ release system for continuous O₂ release to promote cardiac cell viability and also to repair infarcted cardiac tissue.²⁷ Touri et al. demonstrated 3D-printed calcium peroxide and polycaprolactone-based O₂-releasing scaffolds for bone tissue engineering. Their system showed sustained O₂ release over a 10-day period and improved osteoblast cell viability and proliferation under hypoxic conditions.¹⁹ In another study, McQuilling described sodium percarbonate (SPO) and calcium peroxide (CPO)-based silicone films or alginate microspheres as O₂-releasing materials to protect islets during and after transplantation and to prevent cell death under hypoxic conditions.²⁸

Besides providing sufficient and controlled O₂ to engineered tissues, another critical issue that must be considered for tissue engineering purposes is site-specific O₂ release, such that the O₂ is delivered where it is most needed (defect tissue). As such, increasing interest has been paid to injectable O₂-generating biomaterials. Such systems can provide O₂ to cells at the affected part of the tissue while causing no harm to the normal tissue at the site of implantation.

In this context, here we describe a new injectable O₂-generating 3D material prepared by using an organic peroxide, namely benzoyl peroxide (BPO), and Laponite incorporated into an alginate hydrogel. Our injectable O₂-generating biomaterial showed prolonged O₂ delivery for over a period of 14 days and allowed for enhanced cell proliferation under hypoxic (1% O₂) and normoxic (21% O₂) conditions, indicating that our new O₂-generating biomaterial can release sufficient oxygen to cells within the 3D network of the biomaterial to support cells’ continued metabolic activities. This study demonstrates for the first time the potential of organic peroxide-based O₂-generating 3D biomaterials to preserve the survival and to enhance the proliferation rate of cells in 3D an alginate/Laponite nanocomposite hydrogel network. Furthermore, we found that this system resulted in better cell survival for healthy fibroblast cells as compared to malignant Colo 818 cells both under hypoxic and normoxic conditions.

2. MATERIALS AND METHODS

2.1. Materials

Benzoyl peroxide, calcium D-glucanate monohydrate, alginic acid sodium salt, paraformaldehyde (PFA), Triton™ X-100, and albumin from bovine serum lyophilized powder, ≥96% were purchased from Sigma-Aldrich. Laponite powder was obtained from (Laponite RD) Kremer Pigmente GmbH & Co-KG, Germany. Toluene was purchased from Merck. 4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was acquired from Polysciences Europe GmbH. Phalloidin Alexa Fluor 488 was purchased from Invitrogen. The RPMI 1640 cell medium [supplemented with 1% (v/v) penicillin/streptomycin, 2% (v/v) L-glutamate, and 10% (v/v) fetal bovine serum (FBS)], phosphate buffered saline (PBS), ethylenediaminetetraacetic acid (EDTA) 1% in PBS, without Ca²⁺/Mg²⁺, penicillin/streptomycin, L-glutamate, and FBS were obtained from Biochrom, Germany. Primary dermal fibroblasts: normal, human, adult cells were obtained from ATCC. Human Colo 818 (malignant melanoma) cells were purchased from DSMZ.

2.2. Fabrication of oxygen-generating AlgL, ¹BPO-AlgL and ³BPO-AlgL hydrogels and scaffolds

First, three stock solutions were prepared. Alginate (3% wt/v) and calcium D-glucanate monohydrate (1% wt/v) stock solutions were prepared separately in double-distilled water. The BPO (50% wt/v) stock solution was prepared in toluene. Then calcium D-glucanate monohydrate was first mixed with Laponite (30% wt/v), and the formed suspension was sonicated for 1 min. Thereafter, alginate was added into this suspension (1:1) to obtain AlgL hydrogel. In order to generate ¹BPO-AlgL and ³BPO-AlgL hydrogels, alginate was first mixed with different concentrations of BPO, and then the final solution was mixed with the calcium D-glucanate monohydrate/Laponite suspension and kept at room temperature until the toluene was evaporated. The final concentration of BPO in ¹BPO-AlgL and ³BPO-AlgL hydrogels was 1% and 3% (wt/v), respectively. Subsequently, AlgL, ¹BPO-AlgL and ³BPO-AlgL hydrogels were frozen at −20 °C for 24 h and then lyophilized in a freeze-dryer for 24 h to obtain AlgL, ¹BPO-AlgL and ³BPO-AlgL scaffolds.

2.3. 3D printing of AlgL, ¹BPO-AlgL and ³BPO-AlgL hydrogels

The prepared AlgL, ¹BPO-AlgL, and ³BPO-AlgL were printed into grid-like structures using an INKREDIBLE 3D bioprinter (Cellink, Sweden). For printing, we used a 3 cc syringe barrel and a 0.41 mm needle (LOCTITE Dispense Needle Type:97224). The grid structure had dimensions of ca. 22 mm in length, 22 mm in width and ca. 2 mm in height. The speed of the syringe was 80 mm/s, and the injection head speed was 10 mm/s. The extrusion pressure level and the temperature were 60 kPa and 25 °C, respectively.

2.4. Preparation of ⁶BPO, ¹BPO and ³BPO on cell culture plates

Different concentrations of BPO (0%, 1% and 3% wt/v) in ethanol were placed on cell culture plates and dried at room temperature to coat the cell culture plates’ surfaces.

2.5. Oxygen release behaviour

The oxygen-releasing kinetics of media (¹BPO), ¹BPO, ³BPO, AlgL, ¹BPO-AlgL, and ³BPO-AlgL were measured using an oxygen sensor (OXY-1 SMA trace with PST3 sensor spot, Presens GmbH). All samples were incubated at 37 °C, under dark, in serum-free cell culture media in a cell culture plate in a hypoxia box that was flushed with a gas mixture of 1% O₂ + 5% CO₂ + 94% N₂ (hypoxia condition).

2.6. In vitro cell culture studies

The cells were carefully thawed and suspended in cell culture media (RPMI 1640) containing 10% FBS. Then, cells were separately seeded onto the samples in cell culture plates and covered with the cell culture media and incubated for 1 day and 7 days at 37 °C under hypoxic (1% O₂) and normoxic (21% O₂) conditions. After the incubation periods, samples were washed twice with phosphate buffered saline (PBS) to remove non-
adhered cells. Subsequently, the cell viability was measured by PrestoBlue™, metabolic assay.

2.7. Co-staining of cells
The morphology of cells on the samples was determined using a Nikon ECLIPSE Ts2R fluorescence microscope. For co-staining, the cells were seeded separately on the experimental samples and incubated for 1 and 7 days at 37 °C and 5% CO₂. After these incubation times, paraformaldehyde 4% was added to each scaffold and kept for 10 min then washed 2 times with PBS, and then Hoechst 33342 dye was added to each sample for cell nucleus staining. Here, we first made a stock staining solution (16.2 mM), and the stock solution was diluted to 1:2000 in PBS before being added to the cells (we added just enough to cover the cells); cells were incubated at room temperature for 10 min, and then they were washed again twice with PBS. Afterwards, samples were kept in 0.1% Triton X-100 in PBS for 10 min at room temperature, cells were washed 3 times with PBS, and then they were co-stained for f-actin by diluting 5 μL methanolic stock solution (6.6 μM) of Phalloidin Alexa Fluor 488 into 200 μL of PBS containing 3% bovine serum albumin (BSA); after adding the co-staining solution to the cells, they were then kept overnight at room temperature and stored in the dark. After that, the samples were washed 2 times with PBS.

2.8. Characterization
Scanning electron microscopy (SEM) using a Zeiss 1540 EsB dual beam focused ion beam/field emission was performed to observe the cross-sectional morphology of the scaffolds. Nikon ECLIPSE Ts2R fluorescence microscopy was used to show the morphology of fibroblast cells on the samples. Christ Alpha 1-2-LD plus freeze dryer, was used to produce porous hydrogel scaffolds. Cell viability (PrestoBlue assay) was measured using a Tecan Infinite® PRO. Rheological measurements were done using an MCR 302 rheometer (Anton Paar, Ashland, VA, USA) with a 25 mm diameter parallel-plate geometry measuring system. Mechanical properties of samples were analysed using Zwick, type 066590. All tests were done in triplicate and all results are shown as mean ± standard deviation. Also, an ANOVA was performed to determine any significance in the observed data, and p ≤ 0.05 was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Preparation and characterization of O₂-generating biomaterials
The O₂-generating biomaterials were prepared by incorporating different concentrations (1% and 3% wt/v) of BPO into Laponite-embedded Alginate hydrogels (AlgL). BPO belongs to the organic peroxide family and is commonly used for external treatment of acne due to its antimicrobial properties. In contact with skin it is suggested that BPO slowly breakdowns to benzoic acid and oxygen (Fig. S1). The oxygen produces reactive oxygen species that oxidize bacterial proteins. Additionally, under heat (ca. 60 °C), BPO can undergoes thermal homolysis and decomposes to two phenyl radical and CO₂.
One advantage to the use of BPO over inorganic peroxides is that BPO is soluble in organic solvents such as ethanol. Therefore, one can achieve a homogeneous distribution of BPO within the biomaterial, which is crucial for the uniform release of oxygen from the 3D biomaterial scaffold. The 1BPO-AlgL and 3BPO-AlgL hydrogels were printed into 3D grid-like structures to demonstrate their injectability (Fig. 1). We observed that incorporating BPO into AlgL slightly improved the printability of the hydrogels.

Next, we determined the impact of BPO on the degradation, porosity, swelling, and mechanical properties of 1BPO-AlgL and 3BPO-AlgL scaffolds (Table S1-S3, Fig. 2-4). The prepared 1BPO-AlgL and 3BPO-AlgL hydrogels were freeze-dried to achieve porous scaffolds, since porosity is crucial for the efficient diffusion of nutrients and oxygen to cells inside 3D scaffolds (Fig. 2). The SEM images showed that all scaffolds were highly porous, and the porosity of AlgL was reduced by the incorporation of BPO (Fig. 2, Table S1). The swelling ratio (Table S2) of AlgL, 1BPO-AlgL, and 3BPO-AlgL hydrogels decreased with increasing BPO in the AlgL network due to the hydrophobic nature of BPO, which reduces the diffusion of water inside the 3D network of AlgL scaffolds.

Degradation is another important parameter for engineered biomaterials for tissue engineering applications. The fabricated biomaterials should possess slow degradation with time but should be biostable until cells can regenerate the tissue. The degradation of scaffolds (Table S3) increased with time and decreased with the concentration of BPO. Again, that is likely due to the hydrophobic nature of BPO, which reduces media diffusion into the scaffolds.

Rheological tests demonstrated that the incorporation of BPO into AlgL improved the shear thinning and viscoelastic properties of AlgL hydrogels (Fig. 3). AlgL, 1BPO-AlgL, and 3BPO-AlgL showed shear thinning behaviour at increasing shear rates. The shear thinning behaviour of the hydrogels also increased when increasing the concentration of BPO in AlgL. All hydrogels showed a higher storage modulus than loss modulus over the range of angular velocities, indicating that they have viscoelastic properties. Similarly, 1BPO-AlgL, and 3BPO-AlgL scaffolds displayed a higher compressive modulus than AlgL, indicating stronger compressive mechanical properties (Fig. 4).

3.2. Oxygen release kinetics of the O2-generating biomaterials

Thereafter, we determined the oxygen release kinetics of the first set of samples [AlgL (control), 1BPO-AlgL, and 3BPO-AlgL], which were all based on 3D AlgL scaffolds, in cell culture media under hypoxic conditions in a hypoxia box that was flushed with a gas mixture of 1% O2 + 5% CO2 + 94% N2. Next, we also performed the same experiment using not 3D but 2D surfaces: These samples were prepared by coating cell culture plates with different concentrations of BPO [media without BPO (0BPO), 1% BPO (1BPO) and 3% BPO (3BPO)]. Together, these two sample formulations allowed us to determine the impact of the 3D AlgL network on the release kinetics of BPO.
3.3. Cell experiments under hypoxic and normoxic conditions

Subsequently, we performed cell experiments under hypoxic and normoxic conditions (Fig. 6). Since BPO has been used for the treatment of acne and applied on skin, in this study we used skin cells. Primary dermal fibroblasts (normal, human, adult) and human Colo 818 (malignant melanoma) cells were seeded onto AlgL, BPO-AlgL, and BPO-AlgL scaffolds and onto the BPO, BPO, and BPO coated cell culture plates to determine the effect of O₂ delivery on the viability of healthy and malignant cells under hypoxic and normoxic conditions.

Our results, determined using the PrestoBlue metabolic assay, showed that for all samples, the viability of both cell types was significantly lower under hypoxia than under normoxia for 1 day and 7 days of incubation. This difference was not significant for Colo 818 cells at 7 days of incubation, probably because malignant cells have a higher tendency to proliferate than healthy cells. Similarly, for the second set of samples (AlgL, BPO-AlgL scaffolds) we observed that there was no significant difference between the viability of both cell types under both hypoxic and normoxic conditions at 1 day of incubation, while at 7 days of incubation there were a greater number of viable Colo 818 cells than fibroblast cells. For example, we found 2.4 and 5 times more viable Colo 818 cells than fibroblast cells in the BPO sample at 7 days of incubation under normoxia and hypoxia, respectively.

However, the results for the first set of samples (AlgL, BPO-AlgL, and BPO-AlgL scaffolds) were more complex. In general, these 3D scaffolds provided higher number of viable fibroblast and Colo 818 cells than the 2D sample set. The reason is because the 3D network of AlgL can mimic the ECM environment and provides more contact points for cells than the 2D surfaces. Furthermore, in the 3D networks, cells had less direct contact with the BPO than they did on the BPO-coated 2D cell culture plate; this likely resulted in better cell adhesion and proliferation.

In addition, we found a significantly higher number of viable cells in the BPO-AlgL and BPO-AlgL scaffolds than in the AlgL scaffold. For example, at 7 days of incubation, the BPO-AlgL scaffold provided 1.8/1.9 and 1.5/1.5 times more viable fibroblast/Colo 818 cells than the AlgL scaffold under normoxia and hypoxia, respectively. Importantly, by incorporating at least 1% BPO in AlgL, the number of viable cells cultured under hypoxic conditions became significantly higher than the number cultured under normoxic conditions. That was more significant for fibroblast cells than Colo 818. This indicates that the detrimental effects of hypoxic conditions can be avoided by incorporating BPO into a 3D network of AlgL, at least for a period of 7 days. This result was not observed for the second set of samples (2D samples), showing that the beneficial effect of O₂-releasing BPO can only be achieved by incorporating the BPO...
into the 3D AlgL network. Interestingly, in BPO-embedded AlgL scaffolds we observed higher cell viability for healthy fibroblast cells than for malignant Colo 818 cells indicating that O$_2$ release from BPO-AlgL led to a decrease in the proliferation of malignant cells while simultaneously leading to an increase in the viability of healthy cells. For example, at 7 days of incubation, we found 2.1.4 times more viable fibroblast cells in the 1%BPO-AlgL than Colo 818 under normoxia/hypoxia conditions. Furthermore, the difference between the effect of the 1% and 3% BPO-formulated samples on cell viability under both conditions and for both incubation times was not significant. Thus, as general cell viability stayed in the same error range regardless of BPO concentration, we suggest that 1% BPO is sufficient for the fabrication of O$_2$-generating biomaterials. The Colo 818 cell viability between the normoxia and hypoxia conditions after the 7 day incubation period was not significantly different. Similar observations were also seen in the literature.\textsuperscript{34,35} Hypoxia causes decreased cell proliferation for most cell types such as embryonic fibroblast, B lymhocytes. However, certain cell types maintain cell proliferation under hypoxia conditions including cancer cells and cardiomyocytes.\textsuperscript{34,35} The reason is attributed to the activation of the Nuclear Factor Activated T cell (NFAT) signalling via hypoxia-inducible factor (HIF)-2.\textsuperscript{35}

Finally, we analysed the morphology of the cells on 0%BPO, 1%BPO, 3%BPO, AlgL, 1%BPO-AlgL, and 3%BPO-AlgL by fluorescence microscopy (Fig. 7). The adhered cells on 0%BPO had a stretched and elongated shape whereas cells on 1%BPO and 3%BPO had a more round and polygonal shape, indicating the non-cell-adhesive surface of 1%BPO and 3%BPO. On the other hand, cells in 3D AlgL, 1%BPO-AlgL, and 3%BPO-AlgL scaffolds formed clusters and had a round morphology indicating that the AlgL scaffolds promoted 3D cell growth.

4. CONCLUSIONS

In this study, we described a new organic peroxide-based oxygen delivery biomaterial that was prepared by using BPO and Laponite incorporated into an alginate hydrogel. Our new O$_2$-generating biomaterial (BPO-AlgL) was injectable and showed sustained release of O$_2$ over a period of 14 days. Our results show that BPO and its incorporation into a 3D network of AlgL have beneficial effects on cell viability under normoxic and hypoxic conditions. BPO-embedded AlgL was able to reduce hypoxia-induced cell death and enhance cell viability by providing sustained O$_2$ within the 3D AlgL scaffold. In addition, BPO-embedded AlgL scaffolds observed higher cell viability for healthy fibroblast cells than for malignant Colo 818 cells. BPO-embedded AlgL scaffolds increased the O$_2$ level in the cells’ environment, which led to a decrease in the proliferation of malignant cells while at the same time resulted in an increase in viability of the healthy fibroblast cells. Therefore, we envisage that our oxygen-generating organic peroxide-based injectable 3D biomaterials have the potential to be used in the next generation of advanced tissue engineering biomaterials.

Conflicts of interest

There are no conflicts to declare.

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