Inkjet Bioprinting of Solid Peroxide Microparticles for Constructing Oxygen-Generating Scaffolds

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Abstract

A major challenge in tissue engineering is to provide oxygen to cells within implanted tissues before vascularization is achieved. To overcome the limitations, oxygen generating materials have been developed to enhance cell proliferation. However, these methods provide an uneven oxygen distribution. Inkjet bioprinting technology is a novel fabrication approach, in which the bioprinter can deposit biomaterials precisely in a designed path. This technology has been applied in this study to construct oxygen-generating scaffolds. In this study we have applied the inkjet printing technology to allocate calcium peroxide (CPO) in a controlled pattern. The CPO was encapsulated with a hydrogel to provide a progressive oxygen release to cells. Oxygenized scaffolds were utilized searching for optimal cell viability in a period of 10 days under hypoxic environment. The results suggested that scaffolds containing CPO increase cell viability and proliferation in comparison to control group (no CPO) under hypoxic environment along 10 days. In consequence, the designed inkjet printing patterns provided a functional oxygen supply to scaffolds.

Introduction

Tissue Engineering (TE) has originated as a possible solution to overcome the actual shortage of transplantable organs. Currently, the transplantation of engineered tissues should be done in relatively thin layers, to allow angiogenesis after the engineered tissue implantation and meet the oxygen and nutrients demand. Vascularization post-implantation is a principal restriction to use engineered thicker-tissues for applications in demanding organs, such as heart and liver. For regeneration of damage tissues, porous scaffolds loaded with living cells and growth factors have been investigated. One of the principal challenges in TE is the difficulty of providing sufficient oxygen to cells within implanted engineered tissues before full vascularization is achieved. At early implantation stage, oxygen supply is limited to adjacent parts of the scaffold, leaving the further parts of it with restricted or no oxygen at all jeopardizing the viability and proliferation of the seeded cells [1]. This has been a critical limiting factor for developing readily large functional tissues for clinical applications [2].

To promote the oxygen diffusion, novel strategies have been investigated to enhance the oxygen delivery for improved cellular viability of the engineered tissues post-implantation. The use of angiogenic growth factors is a strategy to promote the vascularization in the implanted tissues, such as vascular endothelial growth factor, because is a potent endothelial cell-specific mitogen [3]. Despite the successful angiogenic response in tissues, it has a critical limitation, which is the angiogenesis rate constraint, limiting the size of the implant [4]. The implanted tissue needs to resemble a vascularized tissue to prolong the cellular viability, and promote vascularization [5]. Nevertheless, this vascularization method is not possible when the implant is distant to the target tissue. Other authors have been used perfluorocarbons and crosslinked hemoglobin as synthectic oxygen carriers [6, 7]. Previous studies demonstrate that oxygen releasing biomaterials improve cell viability and prevent necrosis under hypoxic environment [8].

The purpose of this study was to construct oxygen generating scaffolds, depositing diverse micro-particles by the inkjet printing technology [9, 10], and provide a sustainable environment to cells at hypoxic conditions.

Materials and Methods

Functional Ink

Calcium peroxide (CPO) powder (Aldrich, MO, USA) was selected as oxygen generating model. CPO generates oxygen when is in contact with water [11]. CPO was suspended in pure ethanol (Aldrich-Sigma, MO, USA) to form the functional ink. Ethanol does not dissolve CPO, and prevents premature oxygen generating from CPO. The powder was deposited into a 50mL tube and mixed with 10mL of the ethanol to obtain a 1% (w/v) suspension. This suspension was mixed with a vortex mixer (Fisher Scientific, PA, USA).

Assistant Ink

A hydrogel was formed with two assistant inks, alginic-acid/ethanol, and calcium chloride/water. Alginic acid (AA) powder (Acros Organics, NJ, USA) was deposited into 50mL tube and mixed with 10mL of the ethanol to obtain a % (w/v) suspension. Calcium chloride (CaCl2) powder (Acros Organics, NJ, USA) was deposited into 50mL tube and mixed with distilled-sterilized water in order to obtain a 1.4 % (w/v) solution.

All solutions were filtered with a 40µm filter (Fisher Scientific, PA, USA) prior to being dispensed in the printing cartridge to reduce clogging conditions during the printing process.

Printing Process

Modifications to the HP printer model 697C (Hewlett-Packard, Palo Alto, CA) were limited to removing the rubber cleaners, which are used to clean the cartridges nozzles, and bypassing the feed page sensor with push button switch.

A black ink-jet cartridges (HP 29) was emptied of its content, thoroughly washed, rinsed with a 70% ethanol solution, and distilled water and dried in a sterilized Labculture® Class II, Type A2 Biological Safety Cabinet (ESCO, PA, USA) before being filled with 100µL of either solution.

Scaffold Construction

The oxygen generating (OG) scaffolds were constructed by printing alternate layers of functional ink, and assistant inks; obtaining composit scaffolds with oxygen generating micro-particles in the base, and encapsulated on top with hydrogel. These OG scaffolds were investigated for oxygen release.

A 96 well-plate (BD Falcon, NJ, USA) was used as a substrate for the OG Scaffolds. The dot size for build the scaffold was of 4mm of diameter, the printer patterns were designed using NX (Siemens, Munich, Germany); five versions of OG scaffolds were printed with different concentrations of functional ink (Table 1): (i) functional ink was printed into the well, creating 4 different versions, 2, 8, 16, 32 over-printings; (ii) assistant inks were printed 32 times each onto the same functional ink pattern printed, and onto a new well in order to have a control sample with only hydrogel; group samples were identified as shown in the table 1. The different CPO printing over passes were used to determine if the amount of CPO had effects on cell proliferation.

Table 1. Scaffold identification

Sample ID	CPO Printing Passes
OGS-0	Non CPO
OGS-2	2
OGS-8	8
OGS-16	16
OGS-32	32

Cell Culturing

Mouse myoblast cell line C2C12 cells were selected as testing model. Other authors demonstrated that C2C12 proliferation rate is sensitive to oxygen saturation levels [12]. The cell density per well plate was measured using an MTS assay. A standard curve for MTS absorbance was established using control samples with known cell concentrations.

Scaffold Evaluation

To evaluate whether scaffolds containing functional ink could enhance cell proliferation, C2C12 were seeded onto negative and positive control scaffold (OGS-0) and oxygenized scaffolds (OGS-2 thru OGS-32) with a cell density of 10,000 cells/scaffold. The seeded scaffolds were placed in a hypoxic incubator (1% O_2, 5% CO_2) (BioSpherix, USA), and positive scaffolds at normoxic incubation incubator (20%O_2, 5%CO_2). All scaffolds were washed with PBS prior to seeding for improved cell seeding efficiency as described elsewhere [8].

The scaffolds were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% FBS (Gibco), 500 U/mL penicillin (Gibco), 500 mg/mL streptomycin (Gibco). In addition, some aliquots of cells contained 100U/mL catalase (from bovine liver, Sigma, USA). Catalase was added to react with hydrogen peroxide byproducts of metabolism and degradation [8]. These scaffolds were cultured up to 10 days in the hypoxic incubator. The culture medium was carefully changed every 3 days using nitrogen (N_2) purged culture medium to prevent re-oxygenation of the medium. The viable cell number in the scaffolds after culturing for 1, 3, 5, 7 and 10 days was estimated by an MTS cell proliferation assay.

Statistics

All results were presented as mean ± standard deviation. The grouped data were statistically compared to analysis of variance (ANOVA) and a two-sample Student's t-test.

Results

Scaffold Construction

Four different cylindrical CPO scaffolds of 4mm diameter were constructed and all of them were encapsulated by the hydrogel that formed by overprinting 32 times the two assistant inks. Scaffolds without CPO were also constructed to act as control and designated OGS-0. Figure 1 shows a photograph of the resulting hydrogel formed by printing the two assistant inks. Figure 2 shows photographs of the printed CPO samples OSG-2 to OSG-32. As can be seen from the photographs, there is an increased deposition of CPO as expected.

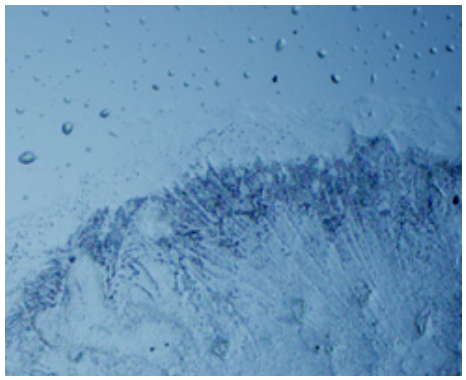

Figure 1. *Hydrogel formation by the bio-printing technology*

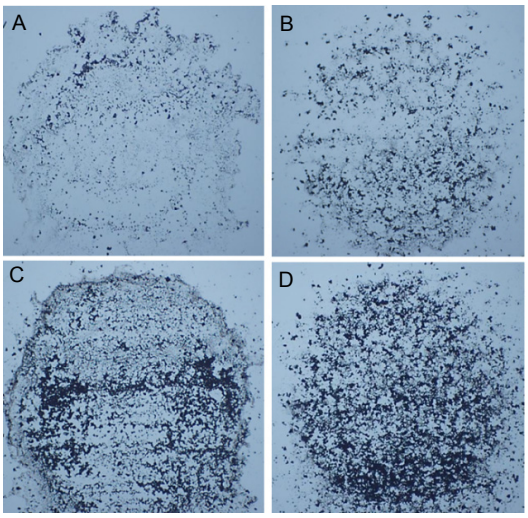

Figure 2*. CPO densities. (A) 2 printing passes, (B) 8 printing passes, (c) 16 printing passes, (D) 32 printing passes.*

Scaffold Evaluation

Figure 3 shows MTS absorbance readings for the C2C12 cells grown on the various scaffolds as function of time. The results at day 1 are comparable for all tests, confirming that the test was initiated under the same conditions for all the scaffolds. This is expected, as the doubling time for C2C12 cells is 2-3 days. The values obtained through the days 3 and 5 were not statistically different (P>0.05). The cells on the different scaffolds proliferated similarly until day five (P>0.05). The scaffolds OGS-2 shows a higher metabolic rate than others at day 7. On scaffolds OGS-32 the cell proliferation decreases after day three. At day 10 the mitochondrial activity decrease dramatically in all the samples placed at hypoxic incubation. The absorbance value in the negative control scaffolds decreased below the initial value, while the cell activity in the scaffolds OGS-2 had an absorbance value above it's initial value but lower than on day 7. The absorbance values for cells growing on the scaffolds OGS-8, OGS-16, and OGS-32 were not statistically different (P>0.05) amongst each other, but significantly higher than cells growing on the negative control, and significantly lower than for cells growing on the OGS-2 scaffolds.

In addition, at day 7 the cells grown on scaffold OGS-2 had approximately 50% more mitochondrial activity than those grown on scaffolds OGS-8, OGS-16, OGS-32, and OSG-0. By the day 10, cells grown on the scaffold OGS-2 had an absorbance number around 330% higher than those grown on negative OGS-0 scaffold, and close to 130% of the activity of cells grown on the scaffolds OGS-8, OGS-16, OGS-32. These results indicate that oxygen-generating scaffolds OGS-2 were more adequate than scaffolds OGS-8, OGS-16, and OGS-32 in supporting cell growth.

Figure 4 illustrates the cell morphology at day 10. The oxygenized scaffolds shown more cells attached to the hydrogel comparing with the negative control scaffold with non CPO. In the group of the oxygenized scaffolds the sample OGS-2 showed more cell proliferation.

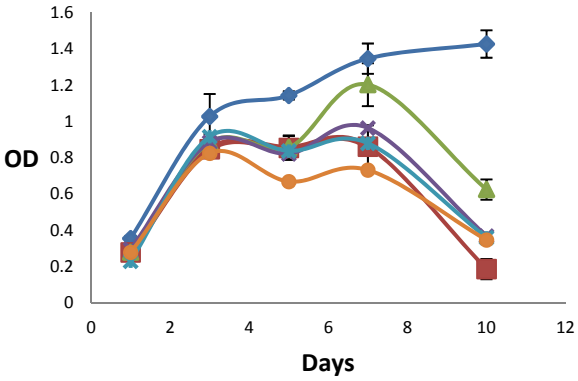

Figure 3. *Absorbance value over time. (◆) Positive Control, (▲) OGS-2, (✕) OGS-8, (✱) OSG-16, (●) OSG-32, (■) Negative control*

Discussion

To obtain an effective supply of cell tissue layers in vitro and to enhance cell survival and growth of transplanted tissue grafts in vivo, a strategy for adequate oxygenation is needed [13, 14]. Despite several methods have been investigated to overcome the vascularization issues such as angiogenic factors, they still far from adequate, especially in large engineered tissues [15]. Therefore, the engineered implanted tissues that exceed the thickness of 150-200μm usually becomes necrotic because of the lack of oxygen in cells [16-18]. In this study, we constructed oxygen-generating scaffolds, which can provide oxygen supply to cells under hypoxic conditions.

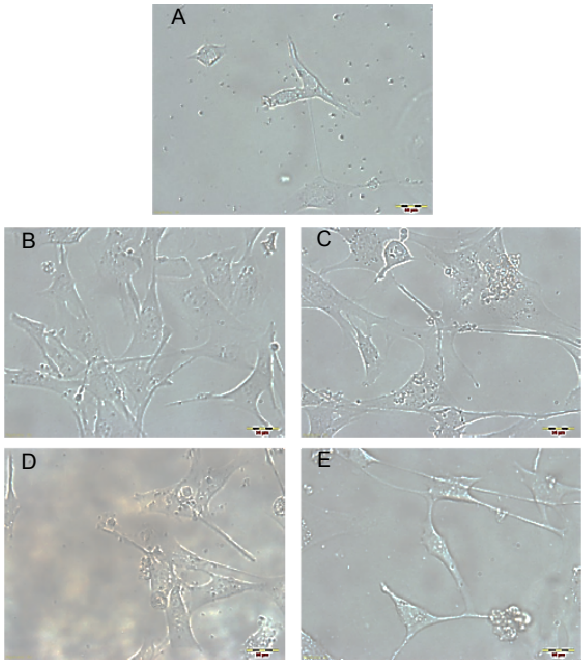

Figure 4*. C2C12 mouse muscle cells morphology after 10 days. (A) Negative control, (B) OGS-2, (C) OGS-8, (D) OGS-16, (E) SGS-32.*

Calcium peroxide was used as an oxygen releasing particle into the scaffold to improve cell viability under hypoxic conditions for 10 days. One concern using the calcium peroxide as oxygen generator, is the presence of hydrogen peroxide that is generated as an intermediate in the calcium peroxide decomposition, and residual reactive oxygen species may be present. Hence, catalase was added to the cells medium to trigger the decomposition of hydrogen peroxide byproducts generated [19].

The results obtained can be attributed to the generation of oxygen from the OGS-2 scaffolds, significantly improve cell viability over negative controls after 7 days. Cell viability of these scaffolds is comparable to positive controls. However, the cell viability of all other scaffolds is below positive control. This is surprising, as these scaffolds contained more CPO and thus were expected to increase the oxygen supply throughout the scaffold. This is attributed to the presence of hydrogen peroxide that is generated as an intermediate in the calcium peroxide decomposition, and residual reactive oxygen species that may be present. The catalase that was added to the growth medium may have been not sufficient to neutralize the peroxide or other reactive species.

The results indicate that scaffolds constructed with oxygen releasing particles in a layer-by-layer fashion may be useful to extend cell viability of engineered cell-tissue implants until

neovascularization is achieved. However, more studies are needed to pinpoint the exact amounts of CPO and catalase needed to extend culture times to 10 days. At the typical the neo-vascularization rate of 0.5-1mm/day in tissue scaffolds, maintaining cell proliferation in the middle of tissue scaffold for 10 days is typically seen as sufficient for centimeter sized tissue scaffolds [20]. Other factors that could potential affect the oxygen generation include hydration, pH, and other chemical/enzymes present in the in vivo environment.

This study demonstrates that building oxygen generating scaffolds is not the only factor contributing to cell viability as excess oxygen can also interfere with viability. Therefore, more studies are needed to establish design criteria for printed 3D scaffolds that improve cell viability for up to 10 days.

Conclusions

In this study, it has been demonstrated that we can deposit CPO scaffolds in a layer-by-layer fashion. One of the scaffolds released oxygen to improve cell survival and growth under hypoxic conditions demonstrating the utility of the printing technique in determining concentrations of CPO needed. We will use these studies to design larger scaffolds with predictable oxygen release for improved cell viability in large tissue-engineered implants.

Acknowledgements

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Author Biography

Daniel Reyna after he received his B.S. in Mexico (ITCJ), he worked in the industry for 5 years designing safety devices. During his M.Sc. in the University of Texas at El Paso (UTEP), he started to be interested in tissue engineering. Presently he is enrolled in the PhD program of BME at UTEP. He has publications related to organ printing and drug screening. His current research is associated to bioprinting technology and oxygen generating scaffolds.