2014-01-01

Inkjet Bioprinting Of Solid Peroxides For Constructing Oxygen Generating Scaffolds To Improve Cells Viability And Growth Under Hypoxic Environment

Daniel Reyna Soriano
University of Texas at El Paso, dreyna2@utep.edu

INKJET BIOPRINTING OF SOLID PEROXIDES FOR CONSTRUCTING OXYGEN GENERATING SCAFFOLDS TO IMPROVE CELLS VIABILITY AND GROWTH UNDER HYPOXIC ENVIRONMENT

DANIEL REYNA SORIANO

Department of Biomedical Engineering

APPROVED:

Thomas Boland, Ph.D., Chair

Bill Tseng, Ph.D.

XiuJun James Li, Ph.D.

Manuel Miranda-Arango, Ph.D.

Charles Ambler, Ph.D.
Dean of the Graduate School

Dedication

I dedicate my dissertation to my lovely wife, my parents, and my baby who is bringing a new motivation to my life.

INKJET BIOPRINTING OF SOLID PEROXIDES FOR CONSTRUCTING OXYGEN GENERATING SCAFFOLDS TO IMPROVE CELLS VIABILITY AND GROWTH UNDER HYPOXIC ENVIRONMENT

by

DANIEL REYNA SORIANO, M.Sc.

DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Department of Biomedical Engineering

THE UNIVERSITY OF TEXAS AT EL PASO

August 2014

Acknowledgements

First and foremost, I owe my deepest appreciation to Dr. Thomas Boland for his support, guidance, friendship, and encouragement throughout this project. I would also like to express my sincere gratitude to my committee members: Dr. Manuel Miranda-Arango, Dr. XiuJun James Li, and Dr. Bill Tseng.

Additionally, I would like to thank Dr. Maria Yanez, Dr. Jorge Rodriguez, M.Sc. Julio Rincon, and M.Sc. Kabir Bhuyan for assistance throughout this project.

In addition, Dr. Ahsan Choudhuri, and Dr. Richard Schoephoerster for their support and continuous encouragement to let this kind of program continue with such progression within the university.

Finally, I would like to thank the following for providing funding for the completion of this adventure: National Science Foundation, University of Texas at El Paso, Department of Mechanical Engineering, Chihuahua's Government special thanks to M.Sc. Cesar Muñoz. Last but not least, I would like to thank my wife for her unconditional love, support, and motivation to do an extra effort every day.

Abstract

Tissue engineering has emerged as an interdisciplinary field to overcome current challenges for tissue repair or replacement in the human body. In essence, it proposes new medical therapies customized to match the biology of specific patients. The potential benefits offered by tissue engineering have driven scientific inquiry to make it a clinical reality.

One of the current challenges in tissue engineering is to provide oxygen supply to thick tissues as oxygen diffusion is limited to 100 - 200 μm layer of viable tissue. Engineered vascular conduits are applied *in vitro*, providing pre-vascularization to tissues; however, host anastomosis is still a problem. Biomaterials are suggested as another strategy; the aim is to construct scaffolds able to provide oxygen to cells in a controlled manner. Tissues require a controlled progressive oxygen supply, otherwise hypoxia or hyperoxia could induce cell necrosis. Inkjet printing method was developed to dispense living organisms, or biomaterials using a designed pattern. The hypothesis tested in this study is that scaffolds can be developed to provide a controlled oxygen supply thereby enhancing cell viability in hypoxic environments.

Calcium peroxide (CaO_2) was selected as oxygen generating material. It was encapsulated in alginate hydrogels to provide a continuous oxygen supply. Inkjet printed microparticles of CaO_2 were evaluated under hypoxic environment (0.01 mol/m^3 O_2) in direct contact with culture medium. Samples with a ratio of 2 mg/mL (CaO_2/medium) exhaust the oxygen supply after 30 hours. Oxygen generating scaffolds fabricated with a ratio of 160 mg of alginate per 10 mg of CaO_2 provided a stable oxygen supply in the range of 0.066 – 0.052 mol/m^3 under hypoxic environment. Fibroblast L-cells were cultured for 120 hours on these scaffolds under hypoxia (0.01 mol/m^3, 5% CO_2, 37 ºC) and their viability was evaluated via MTS metabolic assay. Statistical differences between oxygenized scaffolds and negative control scaffolds were observed. Live/Dead assays corroborated these results.

The current study shows a scaffold composed of alginate-hydrogel, and CaO_2 microparticles dispensed by the inkjet printing technology, able to supply oxygen in contact with

culture medium. The oxygen generating scaffold was capable of maintaining cell mitochondrial activity under hypoxic conditions. The inkjet printing method may play an important role to construct complex scaffolds for tissue engineering applications, where the diffusion of oxygen is a constraint for large engineered-tissue implants.

Table of Contents

List of Tables

List of Figures

Chapter 1

Literature Review

1.1 FUNDAMENTALS OF TISSUE ENGINEERING

Tissue engineering (TE) is an interdisciplinary discipline which applies the principles of life science and engineering to overcome current problems of tissue loss or failure, developing functional engineered tissues for clinical applications [1].

Three principal strategies are used to create tissues [2]: (i) the cells substitute strategy, which helps targeted cells to perform a required function accomplished by selective cell isolation and infusion [3, 4]. However, this strategy has the limitation of immunological rejection [5]. (ii) The tissue-induce substance strategy, which creates new tissue by selection and administration of growth factors [6, 7] coupled with a targeted delivery [8]. (iii) The biomaterial scaffold strategy, in which cells and biomaterials are implanted into the host allowing permeation of oxygen, nutrients, and carbon dioxide (CO_2) [9, 10]. This strategy may also include extracorporeal devices [11]. In a common approach for using biomaterials scaffolds, cells are seeded onto scaffolds under appropriate conditions and subsequently implanted into the body. The biomaterials selection for scaffolds depends of the requirements of the tissue to be repaired or replaced, such as mechanical and chemical properties; these biomaterials can be natural (alginates) and/or synthetic (polymers) [12]. Immunosuppressive drugs [13] or autologous cells may be used within scaffolds to prevent immunological rejection [14]. In all the cases, the strategies are designed to repair or regenerate a tissue structure, and restore some function. Figure 1.1 shows several strategies of tissue engineering.

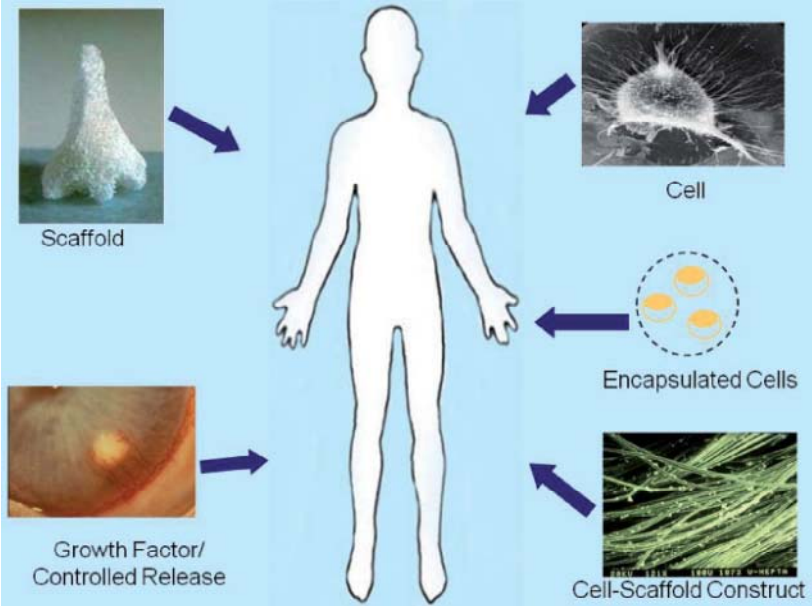

Figure 1.1. Tissue engineering strategies [15]

At the present, TE has provided promising approaches, which includes: (i) the creation of functional crafts for repairing or replacing damaged tissues [16, 17], (ii) development of complex biomaterial 3D-scaffolds for construction of artificial organs [18, 19], and (iii) the creation of engineered tissues models for drug screening [20].

Although, there are important advances in the field, enormous challenges remain; they include: to ensure adequate oxygen supply to thick engineered tissues; to enhance scaffold performance; to develop highly biocompatible materials; and recapitulate the appropriate tissue architecture [21].

1.2 TISSUE ENGINEERING SCAFFOLDS

Different strategies have been developed for constructing engineered tissues using biomaterials-scaffolds. They serve as a synthetic extracellular matrix that provide support for growing cells and mimic the desired tissue architecture [22]. Some scaffolds parameters are porosity, elasticity, stiffness, substance, and specific topography. The scaffolds need to be degradable, because the cells create their own extracellular matrix, and the new tissue is integrated progressively with the surrounding host tissue [18, 23, 24]. Poly (lactide-co glycolide)

(PLG) is a hydrolytically degradable scaffold material; one of the few approved by the FDA for clinical applications [25-27]. PLG is a hydrophobic material, and a solvent such as chloroform is needed during the forming process, requiring extensive cleaning and sterilization before use for cell attachment and growth [28]. Another class of scaffolds is made from hydrogels, hydrophilic water swollen materials that are of natural or synthetic origin. Most hydrogel scaffolds are biodegradable and have similar initial mechanical properties as the tissues in which they are implanted [29].

The mass transport through the scaffold is critical in tissues, because the constant need of gases, proteins, nutrients and waste products. The principal mass transport property of interest is the oxygen diffusion throughout the scaffold to avoid insufficient oxygen levels within the engineered tissue. Investigations were made using oxygen generating systems, [30, 31] however, some limitations are still remaining, including the control of ideal oxygen concentrations for cells, and the control of the 3D oxygenized-scaffold architecture.

1.3 HYDROGELS AS SCAFFOLDS FOR TISSUE ENGINEERING

Hydrogels may be used to create engineered scaffolds using natural or synthetic biomaterials [32-35]. The advantage of using synthetic hydrogels is that material properties can be controlled. Examples of synthetic materials for hydrogel formation are: poly vinyl alcohol (PVA), poly ethylene oxide (PEO) and poly acrylic acids (PAA). Natural hydrogels are used for scaffolds construction, because their similarity to tissues. Examples of natural hydrogels include: collagen, hyaluronic acid, chitosan, and alginate. This literature review is focused on natural hydrogels because they have more prevalence in tissue engineering applications.

1.3.1 Naturally Hydrogel Materials

Hydrogels formed from natural biomaterials have similar properties to the native extracellular matrix (ECM); hence, they are used for different tissue engineering applications such as bone scaffolds and skin repair. Most natural derived hydrogels are linear polysaccharides [37]. As an example, hyaluronic acid (HA) present in animal tissues is widely used as TE

scaffold. Collagen is another natural material used as hydrogel scaffold, and is present in the 25% of mammalian tissue [36]. HA and collagen are two examples of hydrogel scaffolds utilized for TE applications with successful *in vivo* results [38, 39].

1.3.1.1 Collagen

Collagen has appropriate properties for tissue engineering applications. Collagen is a protein, and is present in mammalian tissues as the main structure of ECM. Collagen exists in different types; however, all collagens have the same structure of three polypeptide chains which form a three-stranded configuration. The strands of the collagen have the property to form fibers by self-aggregation [36]. Moreover, mechanical properties of collagen can be improved with chemical cross-linkers such as formaldehyde or carbodiimide [40, 41], using physical treatments including heat, UV light, freeze-drying [40, 42], and combining it with others biomaterials including chitosan, PLG, poly lactic acid (PLA) and HA [40, 41, 43, 44]. The degradation of collagen scaffolds can be locally controlled by cells seeded, because it is degraded by collagenases [36].

1.3.1.2 Hyaluronic Acid (HA)

HA is the only type of glycosaminolglycan (GAG) that is non-sulfated, and is found in mammalian fluid and tissue. HA is elemental for skin in tissue repairing, because it promotes cell proliferation and migration. HA can be formed to hydrogel by different methods, including covalent crosslinking [45, 46], esterification [47], and annealing [48]. Hyaluronidase catalyzes the hydrolysis in HA, inducing a natural degradation in the human body.

1.3.1.3 Chitosan

Chitosan is used in different biomedical applications, because its biocompatibility, and can be naturally degraded *in vivo* by the enzyme lysozyme. Chitosan is a linear polysaccharide that can be obtained by reacting shrimp shells with the alkali sodium hydroxide [49]. Chitosan needs to be dissolved first in dilute acids to form the gelation, which can be induced by increasing pH or extruding the solution [50, 51]. Chitosan derivatives can be formed into gels by

several methods, including glutaraldehyde crosslinking [52, 53], ultra violet radiation [54], and freeze-drying [49, 51].

1.3.1.4 Alginate

Alginate hydrogels are used as scaffolds for tissue engineering applications in particular for bone. The advantage of this material is that gelation can be controlled, and it is highly biocompatible. This material is an anionic polysaccharide copolymer, which is obtained from brown seaweed and bacteria [56]. The monomer M-residues and G-residues of alginate appears consecutive or as interchanging blocks [56, 57]. The particular sequential arrangement of the monomers is affected by the species of the seaweed, its origin and age [56]. Gelation occurs when alginate interact with divalent cations including Ca^{2+} and Ba^{2+}. The hydrogel mechanical properties can be controlled by selecting species with varying the monomers (G and M) ratios and molecular weight [58]. Since alginate hydrogels are only physically crosslinked, they are subject to uncrosslink by ion exchange, resulting in loss of strength over time [59]. Alginate may be synthesized to be hydrolytically degradable by partial oxidation, and a derivative such as polyguluronate [60] creating oxidized alginate [61] and PLG [62].

1.4 STRATEGIES TO PROMOTE VASCULARIZATION WITHIN ENGINEERED TISSUES

Supply of adequate amount of oxygen to the engineered scaffolds is crucial to maintain the cells viability within the scaffold. The oxygen diffusion into tissues is maximum 200 μm [63, 64], hence hypoxic conditions are established within the tissue microenvironment at distances above 200 μm from blood vessels. To overcome this limitation, different strategies are used to incorporate oxygen and improve viability of implanted cells; these include: (i) angiogenic growth factors [65], (ii) synthetic oxygen carriers [66], and (iii) oxygen generating materials [30, 31].

1.4.1 Angiogenic Growth Factors

Angiogenesis is defined as a physiological process where new vessel formation occurs from a preexisting vascular network [67]. The sequence of angiogenesis take place in the following stages: (1) capillary wall degradation, (ii) cell migration, (iii) branch point formation in vessel wall, (iv) new formation arrangement to form a central lumen, and (v) new branched network formation by anastomosis [68]. Direct or indirect the vascularization in vivo is orchestrated by angiogenic growth factors which are present in the natural wound healing, tissue regeneration, and organ growth. There are four principal groups of angiogenic growth factors, these include, epidermal growth factors, fibroblast growth factors, platelet-derived growth factors, and transforming growth factors [69].

1.4.2 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) induces angiogenesis *in vivo* by activating endothelial cells. Cardiac myocytes and retinal cells discharge four isoforms of VEGF having 121, 165 and 206 amino acids [70, 71]. The level of VEGF is increased when tissue is exposed to hypoxic environment *in vivo* as demonstrated by cardio myocytes that showed high VGEF expression under ischemic conditions [72].

1.4.3 Fibroblast Growth Factors

The fibroblast growth factor family is divided in four groups that promote vascularization to fibroblast cells, including FGF-1, FGF-2, FGF-4 and FGF-5 [73]. Acidic FGF and basic FGF are the most characterized factors respective with angiogenic potential that are found in ECM [74]. The four groups of the FGF family induce mitogen stimulus in different paths, stimulating endothelial cells to express urokinase-type plasminogen activator (uPA), and collagenase [75]. Endothelial cell proliferation and migration is promoted by fibroblast growth factor [76, 77]. Acidic FGF and basic FGF promote cell division for smooth muscles cells [78]. Acidic FGF produces mitogenic stimulus to cardiomyocytes and induces capillary formation [79].

1.4.4 Platelet-Derived Growth Factor

Platelet-derived growth factor (PDGF) is found in platelets, monocytes, macrophages smooth muscle cells, and endothelial cells. PDFG produces mitogenic stimulus to fibroblast, and smooth muscle cells that subsequently induces VEGF expression [73, 77]. Transforming growth factor beta-1 induces the expression of PDGF [80]. PDGF released by endothelial cells is related to the pericytes wrapping, and endothelial cell differentiation [77].

1.4.5 Transforming Growth Factor-Beta

Transforming growth factor-β (TGF-β) is a homodimer found as one of five isoforms (β1, β2, β3, β4 and β5). TGF- β1 is released from cells that are triggered by plasmin. TGF- β1 decreases endothelial cells proliferation *in vitro* [77], but promotes new vessels formation *in vivo* [82]. TGF- β1 induces angiogenesis by different mechanisms promoting several angiogenic growth factors, such as PDGF expression by endothelial cells that subsequent stimulates smooth muscle cells to release VEGF and basic FGF [78]. TGF- β also stimulates the angiogenic reaction of cells to PDGF [83]. TFG- β1 is found in several cells, such as platelets and macrophages [84].

1.4.6 Artificial Oxygen Carries

Artificial oxygen carriers are proposed to improve oxygen delivery to tissues, and also used as blood substitutes. These carriers are designed to dissolve oxygen and CO_2 in large quantities, serving as a vehicle for transporting oxygen and removing waste. Modified hemoglobin and perfluorocarbon emulsions are the main artificial oxygen carries [85, 86]. Modified hemoglobin has a similar oxygen solubility curve as blood, and perfluorocarbon emulsions have a linear oxygen concentration with partial pressure [87]. Modified hemoglobin as oxygen carrier is currently being investigated to improve the oxygen transport to hepatocytes in culture [88, 89].

Perfluorocarbons are applied in cardiovascular surgeries as blood substitutes. The FDA approved several perfluorocarbons systems for clinical applications [90]. Perfluorocarbons made into gels can be applied to wounds acting as protecting covering while delivering oxygen [92]. Moreover, alginate scaffolds containing perfluorocarbons are investigated for creating a suitable microenvironment for cells in tissue engineering applications [93].

1.4.7 Oxygen Generating Materials

Solid peroxides generate oxygen when reacts with water, such as magnesium peroxide (MgO_2) and calcium peroxide (CaO_2). Oxygen is generated in two steps; first, solid peroxides react with water resulting hydrogen peroxide as product (Eq 1.1 and Eq. 1.2), subsequently oxygen is generated by the decomposition of hydrogen peroxide (H_2O_2) (Eq. 1.3) [94, 95]. Reaction equation is indicated below.

$CaO_2+2H_2O \rightarrow Ca(OH)_2+H_2O_2$ Eq. 1.1

$MgO_2+2H_2O \rightarrow Mg(OH)_2+H_2O_2$ Eq. 1.2

$2H_2O_2 \rightarrow O_2+2H_2O$ Eq. 1.3

CaO_2 is the most efficient of the solid peroxides as oxygen generating systems in comparison with MgO_2, which has the slowest reaction kinetics, because its low solubility in water [96]. CaO_2 is found in commercial formulations at 80% purity while MgO_2 can be found at 25% purity by weight [97, 98]. In addition, CaO_2 has been used as oxygen-generating compound, because the affordability and availability. Another advantage of solid peroxides for tissue engineering applications is their biocompatibility.

Catalase is an enzyme present in mammalian blood and liver that acts as a catalyst to accelerate the decomposition of H_2O_2 into oxygen and water [99, 100, 101]. The mechanism of catalase is not known precisely, but it is estimated that decomposition reaction of H_2O_2 is given by the following equations:

$H_2O_2+2Fe^{3+} \rightarrow O_2+2Fe^{2+}+2H^+$ Eq.1.4

$H_2O_2+2Fe^{2+}+2H^+ \rightarrow 2H_2O+2Fe^{2+}$ Eq.1.5

The presence of H_2O_2 in cells culturing is known to be toxic for cells, hence many investigators have added catalase to the culture medium.

1.4.8 Inkjet Printing for Tissue Engineering Applications

Inkjet printing technology consists of depositing drops of ink by a non-contact reproductive technique that is controlled by a computer to reproduce a designed pattern [102]. This technique has been applied with promising results to biomedical engineering applications, including drug screening, genomics, biosensors, and tissue engineering [19, 103-105]. The modified printers were successfully used for performing different approaches, such as printing self-assembled monolayers, proteins and other molecules [107]. In addition, this technology has been used to place cells and biomaterials into patterns and thus construct TE scaffolds. [108-110] Figure 1.2 shows a hydrogel scaffold constructed by the inkjet printing technology from reference [106].

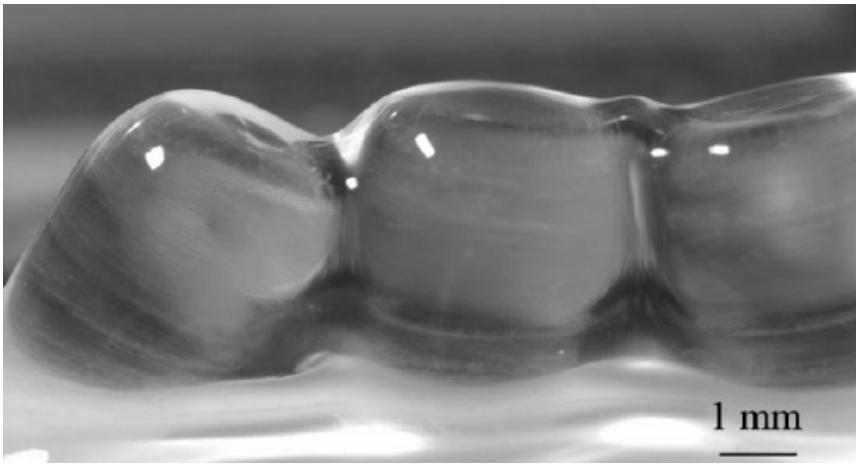

Figure 1.2. Hydrogel Scaffold constructed by inkjet printing technology [106]

1.5 STATEMENT OF THE PROBLEM

Tissue Engineering (TE) has been originated as a solution to overcome the shortage of transplantable organs. Currently, the transplantation of engineered tissues is done in relatively thin layers to allow angiogenesis after implantation in order to meet the oxygen and nutrients demand. The lack of post-implantation vascularization is the principal obstacle to use thick engineered tissues, such as clinical applications in heart and liver. Although porous scaffolds loaded with living cells and growth factors are investigated to create engineered thick tissues; nevertheless, the ability to provide enough oxygen to cells within the scaffold remains the principal challenge. Immediately after implantation, oxygen is limited to the scaffold surface, leaving the interior of the scaffold with restricted or no oxygen at all, thereby jeopardizing the viability and proliferation of the imbedded cells [116]. This has widely been recognized as the critical limiting factor for developing readily large functional tissues for clinical applications [117].

To promote the oxygen diffusion in engineered tissues, several strategies are investigated including the use of growth factors, artificial oxygen carries or oxygen generating materials. The use of angiogenic growth factors, such as vascular endothelial growth factor [72] is restricted by the relatively slow angiogenesis rate, thus limiting the size of the implant [118]. Other strategy is to implant the tissue into similar vascularized tissue for maintaining cell viability and promote vascularization [14]. Nevertheless, this vascularization method is not possible when the implant is distant to the target tissue. Perfluorocarbons and crosslinked hemoglobin as synthetic oxygen carriers [93, 119] are in the early stages of development and some studies demonstrated that they may improve the cell microenvironment [30]. The purpose of this study is to construct oxygen generating scaffolds depositing microparticles by the inkjet printing technology [120, 121], and providing a sustainable microenvironment to cells at hypoxic conditions.

1.6 HYPOTHESIS:

It is hypothesized that inkjet printing technology can be applied to construct oxygen generating scaffolds by printing microparticles of calcium peroxide and hydrogel in a designed pattern. Fabricated scaffolds can provide progressive oxygen supply to cells that are in hypoxic environment, and improve cell viability and growth.

1.7 OBJECTIVE 1

The first objective is to determine how the amount of calcium peroxide microparticles printed relates to the oxygen generation per ml of cell culture medium.

1.7.1 Approach of Objective 1

Modifications to an inkjet printer (HP model 697C, Palo Alto, CA) are limited to removing the rubber cleaners, which is used to clean the cartridges nozzles, and bypassing the feed page sensor with push button switch. Black inkjet cartridges (HP 29) were emptied of their content and washed thoroughly, furthermore, were rinsed with a 70% ethanol solution and distilled water. The cartridges were dried in a sterilized Labculture® Class II - Type A2 Biological Safety Cabinet (ESCO, PA, USA) before being filled with calcium peroxide solutions.

Calcium peroxide (CaO_2) microparticles were suspended in pure ethanol (Aldrich-Sigma, MO, USA) to form the functional ink. Pure ethanol does not dissolve CaO_2 and prevents premature oxygen release. The microparticles were deposited into a 50 mL conical tube, and mixed with 10 mL of the ethanol to obtain a 1% (w/v) suspension. This suspension was mixed with a vortex mixer (Fisher Scientific, PA, USA). The solution was printed onto a glass slide substrate at different densities, and subsequently placed into a nitrogen purged petri dish containing cell culturing medium made of Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), 10% fetal bovine serum (FBS, Gibco), 500 U/mL penicillin (Gibco), 500 mg/mL streptomycin (Gibco), and 100 U/mL of catalase from bovine liver (Sigma, USA). The dish was placed into a hypoxic chamber (BioSpherix, USA), and oxygen concentrations were measured

until they reached background levels of 1%. The oxygen concentration levels were measured in function of time, and the amount of CaO_2 microparticles in the sample provided the information to design the appropriate release system.

1.8 OBJECTIVE 2

Objective 2 is to determine the ratio of calcium peroxide density and hydrogel content to obtain optimal oxygen delivery

1.8.1 Approach of Objective 2

A biodegradable hydrogel was prepared by dissolving alginate (Acros Organics, NJ, USA) in distilled-water at 2% wt. Different volumes of alginate solution were poured onto the calcium peroxide particles samples. After that, 0.25M of $CaCl_2$ was added (Acros Organics, NJ, USA) as described here [112] to promote crosslinking and encapsulating the calcium peroxide particles. The oxygen release as function of alginate gel thickness was obtained.

1.9 OBJECTIVE 3

Objective 3 is to verify that oxygen-generating scaffolds can improve cell viability and growth under hypoxic conditions.

1.9.1 Approach of Objective 3

Fibroblast cell line L-cell was used as testing model. Other authors demonstrated that L-cells have optimal proliferation between 5 and 13% of oxygen concentration [113]. Scaffolds were prepared as in objective 2, and L-cells were seeded with known densities. Three different groups were prepared: negative control, positive control, and oxygen-generating scaffolds. The negative control and oxygen generating scaffolds were placed in hypoxic incubation (1% O_2, 5% CO_2). The positive control scaffolds were cultured at normoxic incubation (20% O_2, 5% CO_2). All scaffolds were washed with PBS for improved cell seeding efficiency [30].

The scaffolds were cultured in DMEM containing 10% FBS, 500 U/mL penicillin, 500 mg/mL streptomycin and 100 U/mL of catalase. The culture medium was changed every day

using nitrogen (N_2) purged culture medium to prevent external oxygenation of the scaffolds. The viable cells number in the scaffolds was assessed every day using MTS assay for a total of 10 days.

1.10 REFERENCES

[1] Heineken FG, Skalak R. Tissue Engineering - a Brief Overview. J Biomech Eng-T Asme. 1991;113:111-2.

[2] Langer R, Vacanti JP. Tissue Engineering. Science. 1993;260:920-6.

[3] Jarrell BE, Williams SK, Stokes G, Hubbard FA, Carabasi RA, Koolpe E, et al. Use of freshly isolated capillary endothelial cells for the immediate establishment of a monolayer on a vascular graft at surgery. Surgery. 1986;100:392-9.

[4] Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7:211-28.

[5] Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood. 2005;106:4057-65.

[6] Duisters RF, Tijsen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, et al. miR-133 and miR-30 Regulate Connective Tissue Growth Factor Implications for a Role of MicroRNAs in Myocardial Matrix Remodeling. Circ Res. 2009;104:170-U61.

[7] Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, et al. Scatter Factor/Hepatocyte Growth-Factor Is Essential for Liver Development. Nature. 1995;373:699-702.

[8] Cao L, Arany PR, Wang YS, Mooney DJ. Promoting angiogenesis via manipulation of VEGF responsiveness with notch signaling. Biomaterials. 2009;30:4085-93.

[9] Lanza RP, Borland KM, Lodge P, Carretta M, Sullivan SJ, Muller TE, et al. Treatment of severely diabetic pancreatectomized dogs using a diffusion-based hybrid pancreas. Diabetes. 1992;41:886-9.

[10] Stevens MM, Marini RP, Schaefer D, Aronson J, Langer R, Shastri VP. In vivo engineering of organs: The bone bioreactor. P Natl Acad Sci USA. 2005;102:11450-5.

[11] Hoerstrup SP, Sodian R, Sperling JS, Vacanti JP, Mayer JE. New pulsatile bioreactor for in vitro formation of tissue engineered heart valves. Tissue Eng. 2000;6:75-9.

[12] Vacanti JP, Morse MA, Saltzman WM, Domb AJ, Perezatayde A, Langer R. Selective Cell Transplantation Using Bioabsorbable Artificial Polymers as Matrices. J Pediatr Surg. 1988;23:3-9.

[13] Murua A, Herran E, Orive G, Igartua M, Blanco FJ, Pedraz JL, et al. Design of a composite drug delivery system to prolong functionality of cell-based scaffolds. Int J Pharm. 2011;407:142-50.

[14] Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB. Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet. 2006;367:1241-6.

[15] Langer R. Perspectives and Challenges in Tissue Engineering and Regenerative Medicine. Adv Mater. 2009;21:3235-6.

[16] Tausche AK, Skaria M, Bohlen L, Liebold K, Hafner J, Friedlein H, et al. An autologous epidermal equivalent tissue-engineered from follicular outer root sheath keratinocytes is as

effective as split-thickness skin autograft in recalcitrant vascular leg ulcers. Wound Repair Regen. 2003;11:248-52.

[17] Wright KA, Nadire KB, Busto P, Tubo R, McPherson JM, Wentworth BM. Alternative delivery of keratinocytes using a polyurethane membrane and the implications for its use in the treatment of full-thickness burn injury. Burns. 1998;24:7-17.

[18] Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB. Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet. 2006;367:1241-6.

[19] Boland T, Xu T, Damon B, Cui X. Application of inkjet printing to tissue engineering. Biotechnol J. 2006;1:910-7.

[20] Hansen A, Eder A, Bonstrup M, Flato M, Mewe M, Schaaf S, et al. Development of a Drug Screening Platform Based on Engineered Heart Tissue. Circ Res. 2010;107:35-U70.

[21] Griffith LG, Naughton G. Tissue engineering--current challenges and expanding opportunities. Science. 2002;295:1009-14.

[22] Kim J, Ma T. Perfusion regulation of hMSC microenvironment and osteogenic differentiation in 3D scaffold. Biotechnol Bioeng. 2012;109:252-61.

[23] Lee K, Chan CK, Patil N, Goodman SB. Cell Therapy for Bone Regeneration-Bench to Bedside. J Biomed Mater Res B. 2009;89B:252-63.

[24] Priya SG, Jungvid H, Kumar A. Skin tissue engineering for tissue repair and regeneration. Tissue Eng Pt B-Rev. 2008;14:105-18.

[25] Kheradmand T, Wang SS, Gibly RF, Zhang XM, Holland S, Tasch J, et al. Permanent protection of PLG scaffold transplanted allogeneic islet grafts in diabetic mice treated with ECDI-fixed donor splenocyte infusions. Biomaterials. 2011;32:4517-24.

[26] Murphy WL, Peters MC, Kohn DH, Mooney DJ. Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering. Biomaterials. 2000;21:2521-7.

[27] Thomson RC, Wake MC, Yaszemski MJ, Mikos AG. Biodegradable polymer scaffolds to regenerate organs. Adv Polym Sci. 1995;122:245-74.

[28] Mikos AG, Thorsen AJ, Czerwonka LA, Bao Y, Langer R, Winslow DN, et al. Preparation and Characterization of Poly(L-Lactic Acid) Foams. Polymer. 1994;35:1068-77.

[29] Faraj KA, van Kuppevelt TH, Daamen WF. Construction of collagen scaffolds that mimic the three-dimensional architecture of specific tissues. Tissue Eng. 2007;13:2387-94.

[30] Oh SH, Ward CL, Atala A, Yoo JJ, Harrison BS. Oxygen generating scaffolds for enhancing engineered tissue survival. Biomaterials. 2009;30:757-62.

[31] Pedraza E, Coronel MM, Fraker CA, Ricordi C, Stabler CL. Preventing hypoxia-induced cell death in beta cells and islets via hydrolytically activated, oxygen-generating biomaterials. P Natl Acad Sci USA. 2012;109:4245-50.

[32] Tamura H, Furuike T, Nair SV, Jayakumar R. Biomedical applications of chitin hydrogel membranes and scaffolds. Carbohyd Polym. 2011;84:820-4.

[33] Rouillard AD, Berglund CM, Lee JY, Polacheck WJ, Tsui Y, Bonassar LJ, et al. Methods for Photocrosslinking Alginate Hydrogel Scaffolds with High Cell Viability. Tissue Eng Part C-Me. 2011;17:173-9.

[34] Kim J, Yaszemski MJ, Lu LC. Three-Dimensional Porous Biodegradable Polymeric Scaffolds Fabricated with Biodegradable Hydrogel Porogens. Tissue Eng Part C-Me. 2009;15:583-94.

[35] Rapaport H, Grisaru H, Silberstein T. Hydrogel Scaffolds of Amphiphilic and Acidic beta-Sheet Peptides. Adv Funct Mater. 2008;18:2889-96.

[36] Lee CH, Singla A, Lee Y. Biomedical applications of collagen. Int J Pharm. 2001;221:1-22.

[37] Smidsrod O, Skjakbraek G. Alginate as Immobilization Matrix for Cells. Trends Biotechnol. 1990;8:71-8.

[38] Ma L, Gao CY, Mao ZW, Zhou J, Shen JC, Hu XQ, et al. Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. Biomaterials. 2003;24:4833-41.

[39] von Heimburg D, Zachariah S, Heschel I, Kuhling H, Schoof H, Hafemann B, et al. Human preadipocytes seeded on freeze-dried collagen scaffolds investigated in vitro and in vivo. Biomaterials. 2001;22:429-38.

[40] Lee CR, Grodzinsky AJ, Spector M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomaterials. 2001;22:3145-54.

[41] Park SN, Park JC, Kim HO, Song MJ, Suh H. Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking. Biomaterials. 2002;23:1205-12.

[42] Schoof H, Apel J, Heschel I, Rau G. Control of pore structure and size in freeze-dried collagen sponges. J Biomed Mater Res. 2001;58:352-7.

[43] Chen GP, Ushida T, Tateishi T. Development of biodegradable porous scaffolds for tissue engineering. Mat Sci Eng C-Bio S. 2001;17:63-9.

[44] Tan W, Krishnaraj R, Desai TA. Evaluation of nanostructured composite collagen-chitosan matrices for tissue engineering. Tissue Eng. 2001;7:203-10.

[45] Oerther S, Maurin AC, Payan E, Hubert P, Lapicque F, Presle N, et al. High interaction alginate-hyaluronate associations by hyaluronate deacetylation for the preparation of efficient biomaterials. Biopolymers. 2000;54:273-81.

[46] Vercruysse KP, Marecak DM, Marecek JF, Prestwich GD. Synthesis and in vitro degradation of new polyvalent hydrazide cross-linked hydrogels of hyaluronic acid. Bioconjugate Chem. 1997;8:686-94.

[47] Mensitieri M, Ambrosio L, Nicolais L, Bellini D, ORegan M. Viscoelastic properties modulation of a novel autocrosslinked hyaluronic acid polymer. J Mater Sci-Mater M. 1996;7:695-8.

[48] Fujiwara J, Takahashi M, Hatakeyama T, Hatakeyama H. Gelation of hyaluronic acid through annealing. Polym Int. 2000;49:1604-8.

[49] Zhang Y, Zhang MQ. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. J Biomed Mater Res. 2001;55:304-12.

[50] Suh JKF, Matthew HWT. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials. 2000;21:2589-98.

[51] Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, et al. Novel injectable neutral solutions of chitosan form biodegradable gels in situ. Biomaterials. 2000;21:2155-61.

[52] Mi FL, Kuan CY, Shyu SS, Lee ST, Chang SF. The study of gelation kinetics and chain-relaxation properties of glutaraldehyde-cross-linked chitosan gel and their effects on microspheres preparation and drug release. Carbohyd Polym. 2000;41:389-96.

[53] Shen F, Cui YL, Yang LF, Yao KD, Dong XH, Jia WY, et al. A study on the fabrication of porous chitosan/gelatin network scaffold for tissue engineering. Polym Int. 2000;49:1596-9.

[54] Ono K, Saito Y, Yura H, Ishikawa K, Kurita A, Akaike T, et al. Photocrosslinkable chitosan as a biological adhesive. J Biomed Mater Res. 2000;49:289-95.

[55] Tomihata K, Ikada Y. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. Biomaterials. 1997;18:567-75.

[56] Johnson FA, Craig DQM, Mercer AD. Characterization of the block structure and molecular weight of sodium alginates. J Pharm Pharmacol. 1997;49:639-43.

[57] Draget KI, Strand B, Hartmann M, Valla S, Smidsrod O, Skjak-Braek G. Ionic and acid gel formation of epimerised alginates; the effect of AlgE4. Int J Biol Macromol. 2000;27:117-22.

[58] Lee KY, Rowley JA, Eiselt P, Moy EM, Bouhadir KH, Mooney DJ. Controlling mechanical and swelling properties of alginate hydrogels independently by cross-linker type and cross-linking density. Macromolecules. 2000;33:4291-4.

[59] LeRoux MA, Guilak F, Setton LA. Compressive and shear properties of alginate gel: Effects of sodium ions and alginate concentration. J Biomed Mater Res. 1999;47:46-53.

[60] Bouhadir KH, Hausman DS, Mooney DJ. Synthesis of cross-linked poly(aldehyde guluronate) hydrogels. Polymer. 1999;40:3575-84.

[61] Bouhadir KH, Lee KY, Alsberg E, Damm KL, Anderson KW, Mooney DJ. Degradation of partially oxidized alginate and its potential application for tissue engineering. Biotechnol Progr. 2001;17:945-50.

[62] Lee KY, Bouhadir KH, Mooney DJ. Degradation behavior of covalently cross-linked poly(aldehyde guluronate) hydrogels. Macromolecules. 2000;33:97-101.

[63] Haraguchi Y, Sekine W, Shimizu T, Yamato M, Miyoshi S, Umezawa A, et al. Development of a New Assay System for Evaluating the Permeability of Various Substances Through Three-Dimensional Tissue. Tissue Eng Part C-Me. 2010;16:685-92.

[64] Muschler GE, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am. 2004;86A:1541-58.

[65] Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. Nat Biotechnol. 2001;19:1029-34.

[66] Kimelman-Bleich N, Pelled G, Sheyn D, Kallai I, Zilberman Y, Mizrahi O, et al. The use of a synthetic oxygen carrier-enriched hydrogel to enhance mesenchymal stem cell-based bone formation in vivo. Biomaterials. 2009;30:4639-48.

[67] Rakusan K. Coronary Angiogenesis - from Morphometry to Molecular-Biology and Back. Ann Ny Acad Sci. 1995;752:257-66.

[68] Sage EH, Vernon RB. Regulation of angiogenesis by extracellular matrix: the growth and the glue. J Hypertens Suppl. 1994;12:S145-52.

[69] Rudkin GH, Miller TA. Growth factors in surgery. Plast Reconstr Surg. 1996;97:469-76.

[70] Banai S, Jaklitsch MT, Shou M, Lazarous DF, Scheinowitz M, Biro S, et al. Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. Circulation. 1994;89:2183-9.

[71] Miller JW, Adamis AP, Aiello LP. Vascular endothelial growth factor in ocular neovascularization and proliferative diabetic retinopathy. Diabetes Metab Rev. 1997;13:37-50.

[72] Ahrendt G, Chickering DE, Ranieri JP. Angiogenic growth factors: A review for tissue engineering. Tissue Eng. 1998;4:117-30.

[73] Moses MA, Klagsbrun M, Shing Y. The role of growth factors in vascular cell development and differentiation. Int Rev Cytol. 1995;161:1-48.

[74] Partanen J, Vainikka S, Korhonen J, Armstrong E, Alitalo K. Diverse receptors for fibroblast growth factors. Prog Growth Factor Res. 1992;4:69-83.

[75] Ware JA, Simons M. Angiogenesis in ischemic heart disease. Nat Med. 1997;3:158-64.

[76] Flaumenhaft R, Abe M, Mignatti P, Rifkin DB. Basic fibroblast growth factor-induced activation of latent transforming growth factor beta in endothelial cells: regulation of plasminogen activator activity. J Cell Biol. 1992;118:901-9.

[77] Hirschi KK, D'Amore PA. Pericytes in the microvasculature. Cardiovasc Res. 1996;32:687-98.

[78] Isner JM. The role of angiogenic cytokines in cardiovascular disease. Clin Immunol Immunopathol. 1996;80:S82-91.

[79] Engelmann GL, Dionne CA, Jaye MC. Acidic fibroblast growth factor, heart development, and capillary angiogenesis. Ann N Y Acad Sci. 1991;638:463-6.

[80] Flaumenhaft R, Rifkin DB. Cell density dependent effects of TGF-beta demonstrated by a plasminogen activator-based assay for TGF-beta. J Cell Physiol. 1992;152:48-55.

[81] Goad DL, Rubin J, Wang H, Tashjian AH, Jr., Patterson C. Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology. 1996;137:2262-8.

[82] Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci U S A. 1986;83:4167-71.

[83] Marx M, Perlmutter RA, Madri JA. Modulation of platelet-derived growth factor receptor expression in microvascular endothelial cells during in vitro angiogenesis. J Clin Invest. 1994;93:131-9.

[84] Yang EY, Moses HL. Transforming growth factor beta 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. J Cell Biol. 1990;111:731-41.

[85] Adlercreutz P, Mattiasson B. Oxygen-Supply to Immobilized Cells .3. Oxygen-Supply by Hemoglobin or Emulsions of Perfluorochemicals. Eur J Appl Microbiol. 1982;16:165-70.

[86] King AT, Mulligan BJ, Lowe KC. Perfluorochemicals and Cell-Culture. Bio-Technol. 1989;7:1037-42.

[87] Spahn DR. Current status of artificial oxygen carriers. Adv Drug Deliver Rev. 2000;40:143-51.

[88] Gordon J, Palmer AF. Impact of increased oxygen delivery via bovine red blood cell supplementation of culturing media on select metabolic and synthetic functions of C3A hepatocytes maintained within a hollow fiber bioreactor. Artif Cell Blood Sub. 2005;33:297-306.

[89] Sullivan JP, Gordon JE, Palmer AF. Simulation of oxygen carrier mediated oxygen transport to C3A hepatoma cells housed within a hollow fiber bioreactor. Biotechnol Bioeng. 2006;93:306-17.

[90] Krafft MP. Fluorocarbons and fluorinated amphiphiles in drug delivery and biomedical research. Adv Drug Deliver Rev. 2001;47:209-28.

[91] Oxynoid OE, Sydliarov DP, Aprosin YD, Obraztsov VV. Application of Fluorocarbon Emulsions as Components of Cosmetics and Medical Ointments. Artificial Cells Blood Substitutes and Immobilization Biotechnology. 1994;22:1331-6.

[92] Magdassi S, Royz M, Shoshan S. Interactions between Collagen and Perfluorocarbon Emulsions. International Journal of Pharmaceutics. 1992;88:171-6.

[93] Khattak SF, Chin KS, Bhatia SR, Roberts SC. Enhancing oxygen tension and cellular function in alginate cell encapsulation devices through the use of perfluorocarbons. Biotechnol Bioeng. 2007;96:156-66.

[94] Northup A, Cassidy D. Calcium peroxide (CaO_2)for use in modified Fenton chemistry. J Hazard Mater. 2008;152:1164-70.

[95] Waite AJ, Bonner JS, Autenrieth R. Kinetics and stoichiometry of oxygen release from solid peroxides. Environ Eng Sci. 1999;16:187-99.

[96] Borden RC, Goin RT, Kao CM. Control of BTEX migration using a biologically enhanced permeable barrier. Ground Water Monit R. 1997;17:70-80.

[97] Cassidy DP, Irvine RL. Use of calcium peroxide to provide oxygen for contaminant biodegradation in a saturated soil. J Hazard Mater. 1999;69:25-39.

[98] White DM, Irvine RL, Woolard CR. The use of solid peroxides to stimulate growth of aerobic microbes in tundra. J Hazard Mater. 1998;57:71-8.

[99] Raducan A, Cantemir AR, Puiu M, Oancea D. Kinetics of hydrogen peroxide decomposition by catalase: hydroxylic solvent effects. Bioproc Biosyst Eng. 2012;35:1523-30.

[100] Schmidtke T, White D, Woolard C. Oxygen release kinetics from solid phase oxygen in Arctic Alaska. J Hazard Mater. 1999;64:157-65.

[101] Chelikani P, Fita I, Loewen PC. Diversity of structures and properties among catalases. Cell Mol Life Sci. 2004;61:192-208.

[102] Mohebi MM, Evans JR. A drop-on-demand ink-jet printer for combinatorial libraries and functionally graded ceramics. J Comb Chem. 2002;4:267-74.

[103] Collier WA, Janssen D, Hart AL. Measurement of soluble L-lactate in dairy products using screen-printed sensors in batch mode. Biosens Bioelectron. 1996;11:1041-9.

[104] Hughes TR, Mao M, Jones AR, Burchard J, Marton MJ, Shannon KW, et al. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat Biotechnol. 2001;19:342-7.

[105] Lemmo AV, Rose DJ, Tisone TC. Inkjet dispensing technology: applications in drug discovery. Curr Opin Biotechnol. 1998;9:615-7.

[106] Wilson WC, Boland T. Cell and organ printing 1: Protein and cell printers. Anat Rec Part A. 2003;272A:491-6.

[107] Roth EA, Xu T, Das M, Gregory C, Hickman JJ, Boland T. Inkjet printing for high-throughput cell patterning. Biomaterials. 2004;25:3707-15.

[108] Boland T, Mironov V, Gutowska A, Roth EA, Markwald RR. Cell and organ printing 2: fusion of cell aggregates in three-dimensional gels. Anat Rec A Discov Mol Cell Evol Biol. 2003;272:497-502.

[109] Mironov V, Boland T, Trusk T, Forgacs G, Markwald RR. Organ printing: computer-aided jet-based 3D tissue engineering. Trends Biotechnol. 2003;21:157-61.

[110] Wilson WC, Jr., Boland T. Cell and organ printing 1: protein and cell printers. Anat Rec A Discov Mol Cell Evol Biol. 2003;272:491-6.

[111] Sinensky MC, Leiser AL, Babich H. Oxidative stress aspects of the cytotoxicity of carbamide peroxide: in vitro studies. Toxicol Lett. 1995;75:101-9.

[112] Atala A, Cima LG, Kim W, Paige KT, Vacanti JP, Retik AB, et al. Injectable alginate seeded with chondrocytes as a potential treatment for vesicoureteral reflux. J Urol. 1993;150:745-7.

[113] Taylor GW, Kondig JP, Nagle SC, Jr., Higuchi K. Growth and metabolism of L cells in a chemically defined medium in a controlled environment culture system. I. Effects of O_2 tension on L-cell cultures. Appl Microbiol. 1971;21:928-33.

[114] Mates JM. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology. 2000;153:83-104.

[115] Cao Y, Mitchell G, Messina A, Price L, Thompson E, Penington A, et al. The influence of architecture on degradation and tissue ingrowth into three-dimensional poly(lactic-co-glycolic acid) scaffolds in vitro and in vivo. Biomaterials. 2006;27:2854-64.

[116] Folkman J, Hochberg M. Self-regulation of growth in three dimensions. J Exp Med. 1973;138:745-53.

[117] Khademhosseini A, Langer R, Borenstein J, Vacanti JP. Microscale technologies for tissue engineering and biology. Proc Natl Acad Sci U S A. 2006;103:2480-7.

[118] Smith MK, Peters MC, Richardson TP, Garbern JC, Mooney DJ. Locally enhanced angiogenesis promotes transplanted cell survival. Tissue Eng. 2004;10:63-71.

[119] Gordon JE, Dare MR, Palmer AF. Engineering select physical properties of cross-linked red blood cells and a simple a priori estimation of their efficacy as an oxygen delivery vehicle within the context of a hepatic hollow fiber bioreactor. Biotechnol Progr. 2005;21:1700-7.

[120] Xu T, Gregory CA, Molnar P, Cui X, Jalota S, Bhaduri SB, et al. Viability and electrophysiology of neural cell structures generated by the inkjet printing method. Biomaterials. 2006;27:3580-8.

[121] Xu T, Jin J, Gregory C, Hickman JJ, Boland T. Inkjet printing of viable mammalian cells. Biomaterials. 2005;26:93-9.

Chapter 2

Correlation of Bioprinting of Solid Peroxide Microparticles and Oxygen Generating

2.1 INTRODUCTION

Oxygen generating materials are suggested for tissue engineering applications to overcome the current problem of hypoxia in engineered tissues [1]. One such class of materials that has received some attention lately is the one of solid peroxides. Calcium peroxide (CaO_2), the most commonly used of the solid peroxides, decomposes into hydrogen peroxide upon exposure to water. The hydrogen peroxide subsequently decomposes into water and oxygen in a second step. The second step requires a catalyst such as catalase, an enzyme founding in the blood and liver. Calcium peroxide was used to oxygenate scaffolds in previous studies, but the oxygen generating particles are not distributed evenly enough to provide a uniform oxygen level [2].

Inkjet printing technology is a fabrication technique, where a modified off-the-shelf printer can print living organisms in a designed pattern [3]. The advantages of this technology are: high through put, drop on demand, variety of materials can be printed, and low cost [4]. This technology has been applied in biomedical engineering with promising results in drug screening, genomics, and biosensors. [5-7]. More recently, inkjet printing technology has been used in tissue engineering applications, where cells and biomaterials were printed to construct scaffolds and cellular structures [8]. In this study, inkjet printing technology is applied to control the dispensing of calcium peroxide microparticles.

2.2 MATERIALS AND METHODS

2.2.1 Printer and Cartridge modifications

The modifications to the HP printer model 697C (Hewlett-Packard, Palo Alto, CA) were limited to removing the rubber cleaners and their respective springs that are used to clean the cartridges nozzles. Moreover, the feed page sensor was bypassed and a push-button switch was added (See figure 3.1). Black ink-jet cartridges (HP 29) were emptied of their content and thoroughly washed with a 70% ethanol solution and distilled water. Then the cartridges were dried in a biological safety cabinet before to being used.

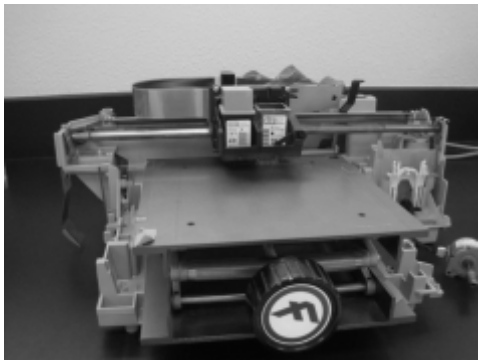

Figure 2.1. Photograph of the modified HP 297C Printer.

2.2.2 Ink preparation

Calcium peroxide (CaO_2) powder (Sigma-Aldrich, MO, USA) was deposited into a 50 mL tube and mixed with 10 mL of 200-proof ethanol (Sigma-Aldrich, MO, USA), obtaining a suspension of 1% wt. Then the suspension was mixed with a vortex mixer (Fisher Scientific, PA, USA), filtered with a 40 μm mesh (Fisher Scientific, PA, USA), and dispensed in the printing cartridge.

2.2.3 Volume printed prediction

In determining the amount of volume being dispensed by the inkjet printer, a given volume of a fully saturated sodium chloride solution (30%) (Acros Organics, Geel, Belgium)

was printed, dried, and weighed. The following patterns were thus printed. As control, a volume of 100 μL was dispensed onto substrates under a conventional micropipette.

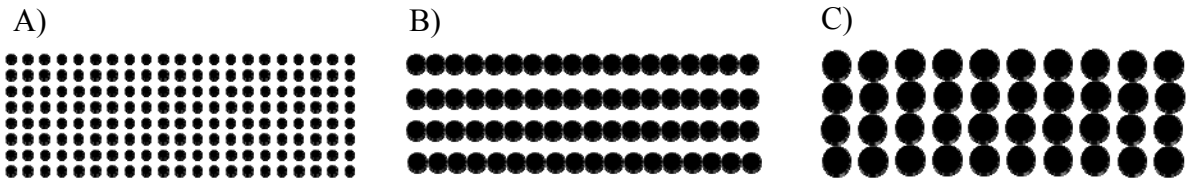

Figure 2.2. Patterns printed for volume determination. . (A) Dot size 0.05", (B) Dot size 0.10", (C) Dot size 0.15"

2.2.4 Printing of designed patterns

The calcium peroxide suspension was printed according to the patterns shown in Figure 2.3, varying the over-printings to deposit a total of 1 mg, 5 mg and 10 mg, onto a 22x22 mm glass slides. The glass slide was pre-heated to 80 ºC to accelerate the solvent evaporation. The 1 mg group was obtained by printing 64 layers, the 5 mg group had 320 layers, and the 10 mg group had 640 layers printed, replacing each cartridge with a new one every 32 layers. After the samples were dried out at room temperature, the weights of the samples were measured.

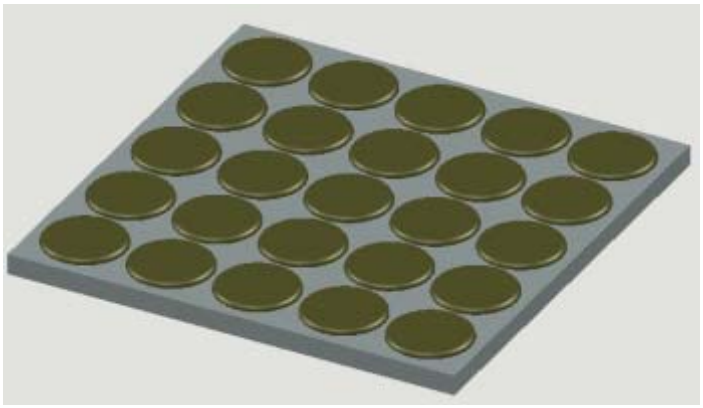

Figure 2.3. Printing Pattern

2.2.5 Oxygen concentration measurements

Culture medium was Dulbecco's modified Eagle's medium, with 10% FBS, 500 U/mL penicillin, 500 mg/mL streptomycin (Gibco, San Diego, CA), and 100 U/mL of catalase (Sigma-

Aldrich, MO, USA). Oxygen concentration was measured over time at hypoxic environments of 0.01 mol using a specially designed glove box system (BioSpherix, Lacona, NY) (see figure 2.4) Media was purged for 24 hours with N_2 and oxygen concentration was confirmed by an oxygen sensor. Negative control samples containing normoxic oxygen concentration levels at 0.18 mol were placed into 50 ml tubes. Continuous oxygen readings were obtained by an oxygen sensor (OAKTON Instruments, IL, USA). The measurements were recorded over time until the media reached background levels of oxygen concentration at 0.01 mol.

To measure the oxygen release rate from the printed calcium peroxide microparticles, three groups were evaluated under hypoxic conditions by placing the samples into 6 well-plates containing 5 ml of purged culture media. The three concentrations of CaO_2 to culture media were: 0.02 %, 0.1% and 0.5%. The oxygen concentration measurements were taken periodically with the sensor until it reached hypoxic levels. A pH-meter was used to measure the pH of the culture media.

Figure 2.4. GloveBox System.

2.3 RESULTS

2.3.1 Obtaining equation of volume printed

The patterns of printed NaCl are shown in Figure 2.5, and the physical dimensions were 0.05, 0.10, and 0.15 inches in diameter according to the software which ultimately corresponded to actual printed areas of 1.24, 5.30, and 11.87 mm^2, respectively. The dispensed volumes were 4.84, 27.76, and 60.16 nanoliters. Data were plotted adding the natural value of 0,0 (area, volume) and different trend lines were draw. A positive correlation was found within the two set of data (correlation = 0.99946), which confirms the direct relation between the area of the dot and the volume dispensed. A trend line was found to be the best line fit (regression line) (R-squared = 99.89) that resulted in a linear equation that describes the behavior of the volume with respect to the printed area. The resulting equation was:

$$Volume\ (y) = 5.1307\ area(x) - 0.4467 \qquad \text{Eq. 2.1}$$

This equation was used to predict the amount of calcium peroxide dispensed per the printing designed pattern.

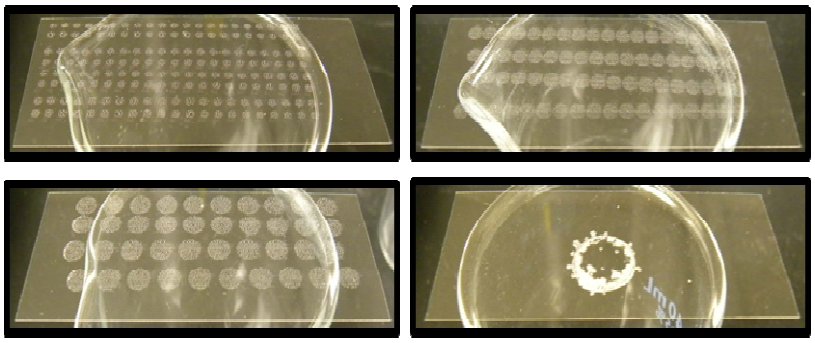

Figure 2.5. Patterns printed for volume determination. (A) Dot diameter 0.050". (B) Dot diameter 0.100". (C) Dot diameter 0.150". (D) Control, 100 µL deposited manually

Sodium chloride solution was found to be an accurate substance to be used as a control for the volume determination test, as its percentile error of 8.99%.

The results of the three different sets of printed CaO_2 particles are shown in table 2.1.

Table 2.1. Mass of printing of pattern layers of CaO_2-Ink

Printing Passes	***Mass predicted (mg)***	***Mass (mg); Average n=3***	***Error (%)***
64	1.0	0.9	10%
320	5.0	4.3	14%
640	10.0	11.6	16%

2.3.2 Oxygen concentration of medium in a hypoxic environment over time

Figure 2.6 shows the oxygen concentration in the media as function of time. Oxygen concentration decreases from normoxic (0.18 mol/m^3) to hypoxic (0.01 mol/m^3) levels in 60 minutes. Hence, this result suggested that cells incubated under those conditions may experiment hypoxia after 1 hour, excluding the oxygen cells consumption. The results show exponential oxygen diffusion to the environment, which is governed by Fick's law, where postulates that diffusion goes from region of high concentrations to region of low concentrations with a magnitude that is proportional to the gradient concentration. The plot was fitted with a logarithmic equation, *y (concentration) = -0.036ln (time) + 0.1499*, which seems to underscore this point.

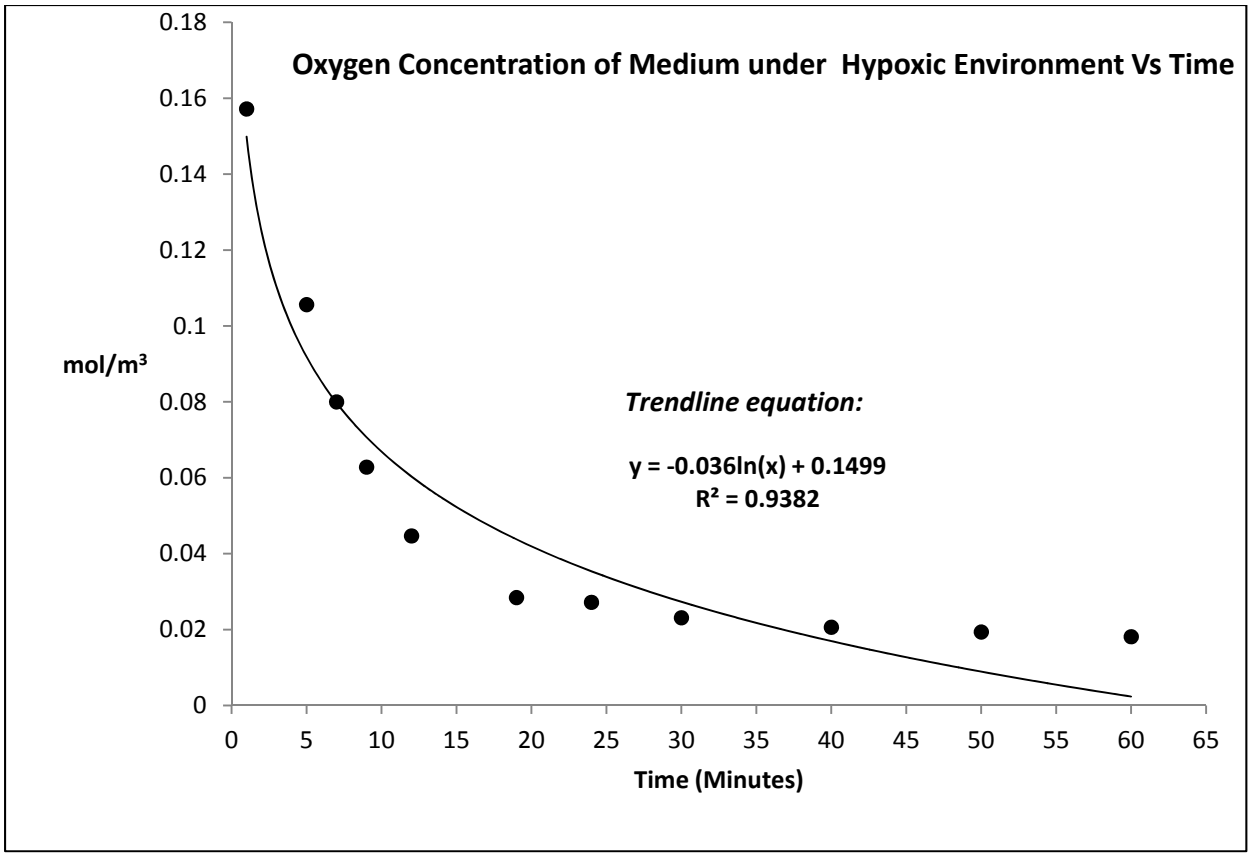

Figure 2.6. Oxygen concentration of medium versus time under hypoxic environment

2.3.3 Correlation of oxygen concentration with calcium peroxide density

Figure 2.7 shows the plotted results of the 0.02 % CaO_2 group in culture medium under hypoxic conditions. The oxygen concentration in the media decreased exponentially. The maximum oxygen concentration of 0.104 mol/m^3 occurred at 9 minutes after exposure to the culture medium. Oxygen concentration reached background levels after 8.5 hours, which is 850% higher than samples without CaO_2.

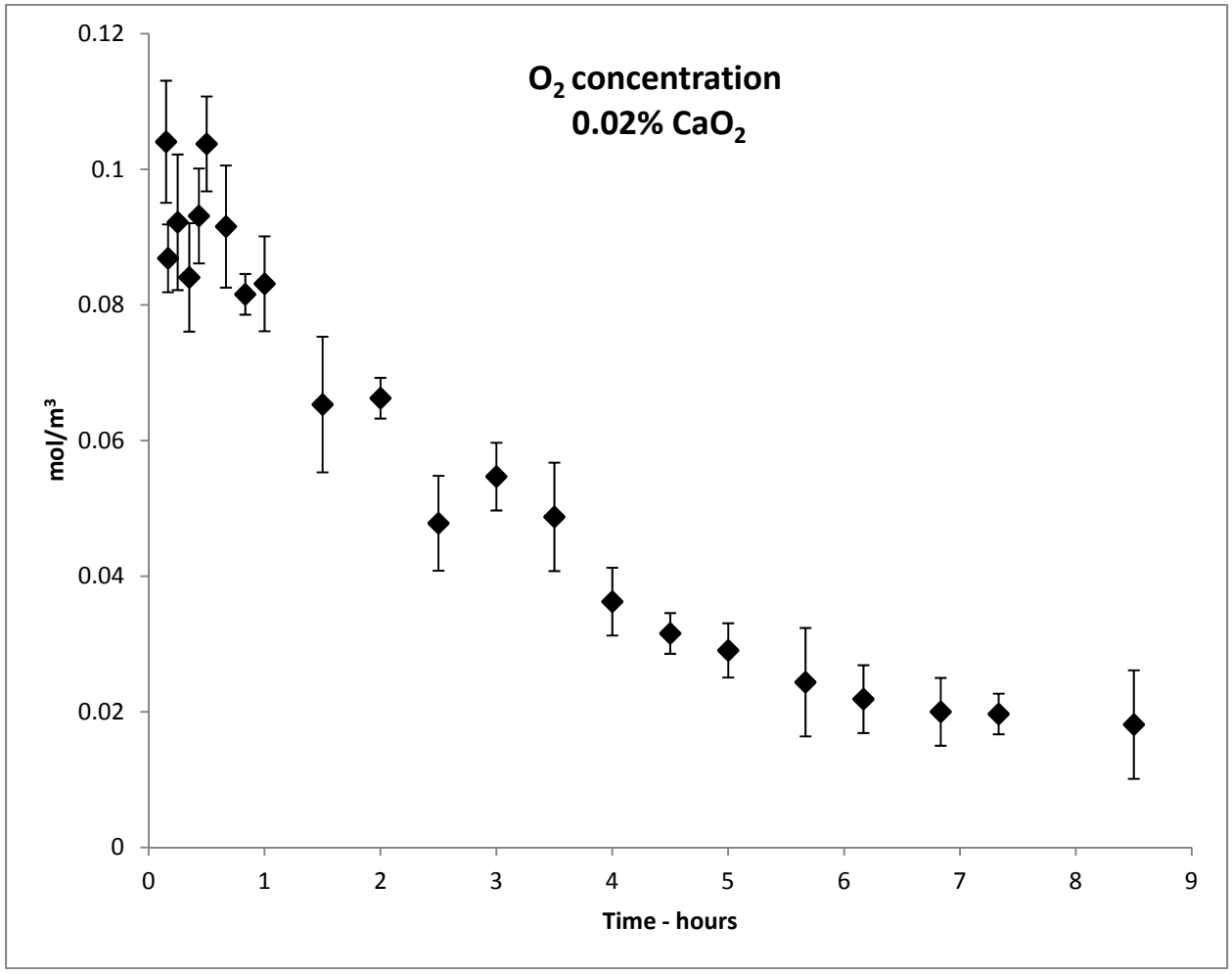

Figure 2.7. Oxygen generations from a 0.02% calcium peroxide solution in culture media (n=3).

Figure 2.8 shows the plotted results of the 0.1 % CaO_2 group in culture medium under hypoxic conditions. The oxygen concentration reached background levels after 25 hours and the oxygen concentration peak was 0.22 mol/m^3 at 7 minutes after exposure to culture medium. The maximum oxygen concentration was two times greater that the group 0.02%, and the oxygen exhaustion were prolonged for 16.5 hours. The oxygen concentration stayed above the hypoxia level of 0.05 mol/m^3 for 21 hours.

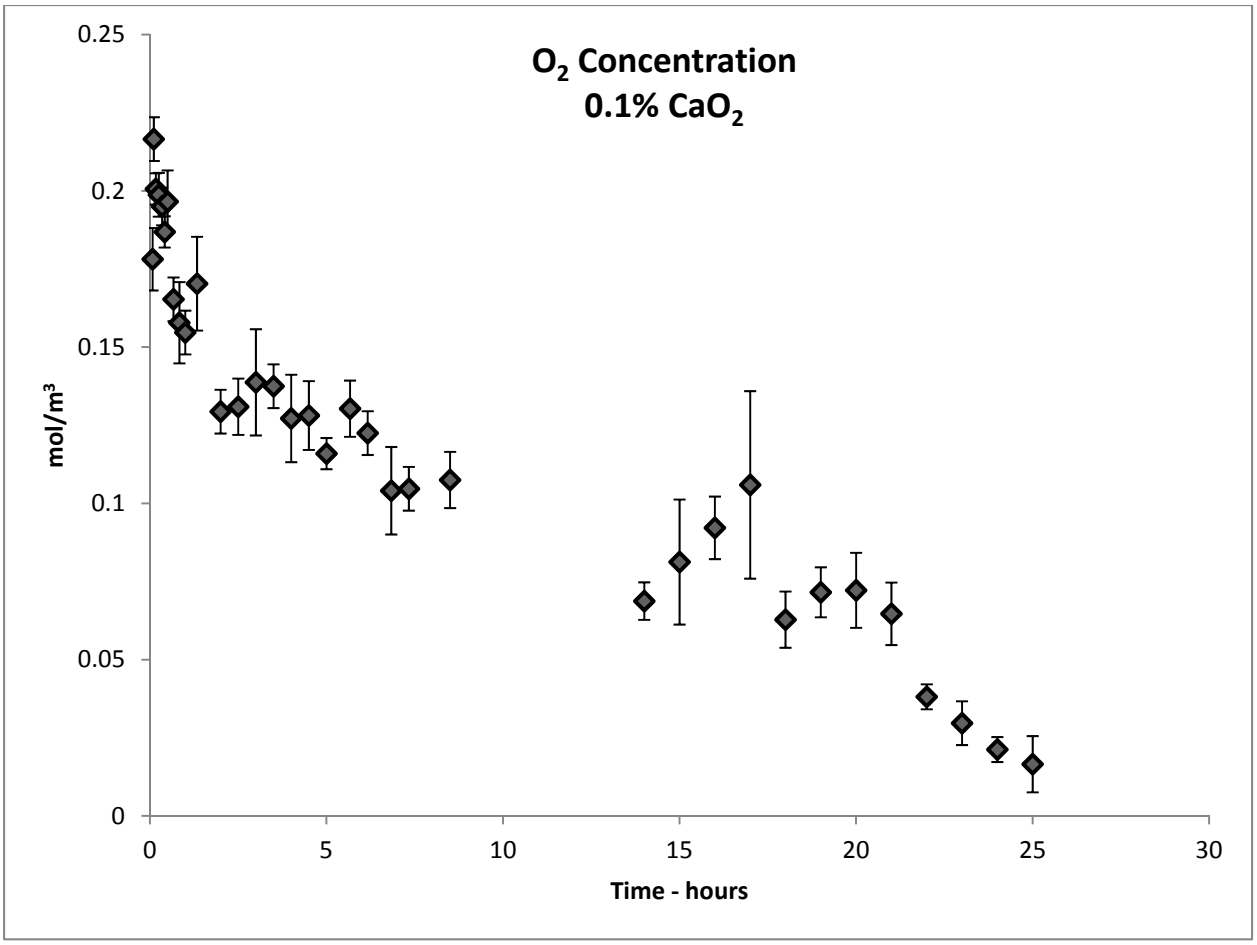

Figure 2.8. Oxygen generations from a 0.1% calcium peroxide solution in culture media (n=3)

Figure 2.9 shows the plot of the dissolved oxygen concentrations for the 0.2% CaO_2 group. The media reached hypoxic levels after 30 hours, and during the initial 15 minutes, the oxygen level was at hyperoxia levels of 0.62 mol/m^3. The pH readings obtained during the study was between 6.79 and 7.27, which is suitable for cell culturing.

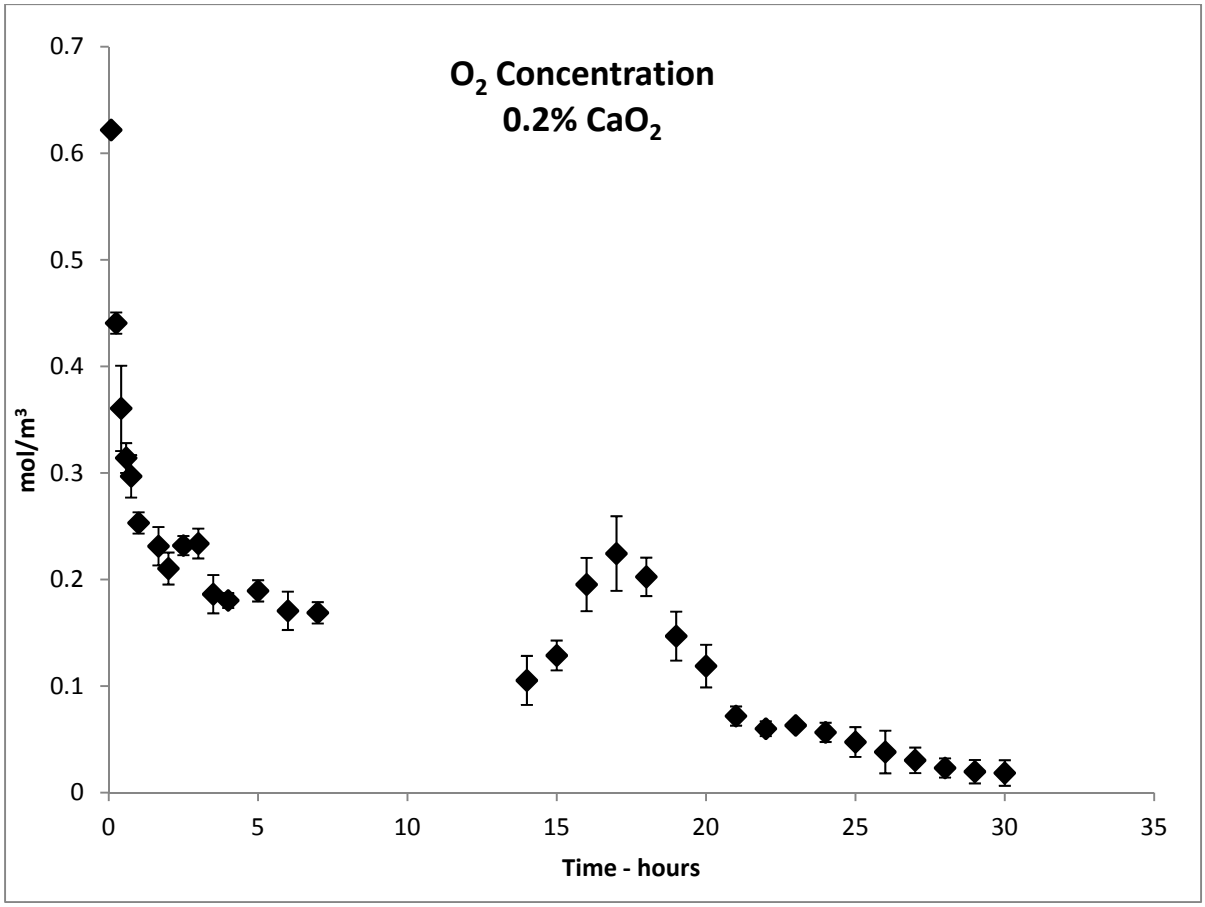

Figure 2.9. Oxygen generations from 0.2% of calcium peroxide solution in culture media (n=3)

2.4 DISCUSSION

Inkjet printing technology is a fabrication technique that has been applied for printing a variety of materials and/or living organisms for different applications [9]. Previous authors demonstrated that this fabrication technique can be used to construct complex hydrogel scaffolds [10]. In this study an equation was obtained to predict the mass dispensing by the modified off-the-shelf printer HP697C. The mass predicted for printing 1 mg, 5 mg and 10 mg of CaO_2 microparticles had a percentage error of 10%, 14% and 16% respectively. This variation may refer to the particles sedimentations, which affect the nozzles during printing process. In a previous study, living organisms were dispensing using inkjet printing technology with a percentage error of 35% [11]. The capability of mass dispensing in the range of milligrams represents an important advantage for biofabrication, where constructing scaffolds composed of different materials with a controlled position is required.

Oxygen concentration of culture medium was evaluated in a hypoxic environment. Oxygen diffusion from the medium to the hypoxic environment is governed by Fick's second law, where oxygen diffusion with time "t" in one dimension from a boundary at "x = 0", and the concentration maintained at a value "n_0" is described below:

$$n(x,t) = n_0 erfc\left(\frac{x}{2\sqrt{Dt\pi}}\right) \qquad \text{Eq.2.2}$$

Where "n_0" is 0.16 mol/m^3, time "t" in seconds, length "x" is 0.005m, D = $3x10^{-9}m^2/s$ [12] and with an "erfc" value of 0.157. The figure 2.10 shows the equation plotted with respect time "t", and the values obtained during the experiment are represented in the same graph. The exponential decay of the oxygen concentration in the media correlates with the oxygen diffusion calculation by Fick's second law equation.

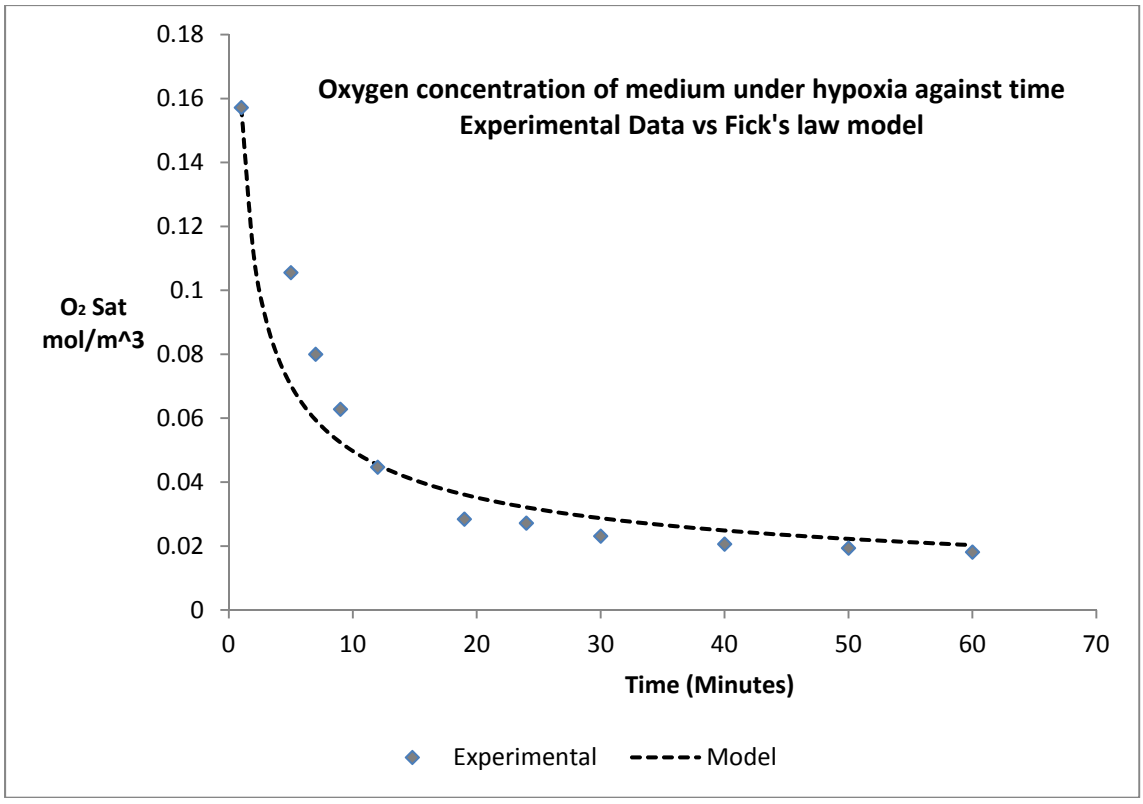

Figure 2.10 Oxygen concentration of culture medium under hypoxia conditions. Experimental data vs Fick's law model data.

Calcium peroxide is used for different applications to release oxygen for sustained time periods in agriculture and aquaculture [13, 14]. Calcium peroxides have been used to generate oxygen in tissue engineering scaffolds, and studies suggest that they are suitable for cell culturing [15]. Calcium peroxide generates oxygen when is in contact with water according to the following equations [16]:

$$CaO_2 + 2H_2O \rightarrow Ca(OH)_2 + H_2O_2 \quad \text{Eq. 2.3}$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad \text{Eq. 2.4}$$

One concern about using calcium peroxide for tissue engineering applications, is that the first decomposition steps is the generation of hydrogen peroxide, leading the presence of reactive

oxygen species. Hence, catalase was incorporated to the cell culture medium to accelerate the reaction of hydrogen peroxide [17].

Inkjet printing technology was used to print calcium peroxide microparticles (< 40 μm), forming three groups of 0.02%, 0.1% and 0.2% solutions (n=3). The theoretical O_2 generations from these groups are shown in table 2.2 below.

Table 2.2. Theoretical Oxygen generation of CaO_2/Medium proportion: 0.02%, 0.1% and 0.2%.

CaO_2 concentration w/V	**Theoretical O_2 generation**
0.02 %	0.77 mol/m^3
0.1%	3.85 mol/m^3
0.2%	7.70 mol/m^3

The oxygen generated by the three calcium peroxide/medium proportions described above was estimated by numerical integration (trapezoidal rule) as follows:

$$\int_a^b f(x)dx \approx \frac{a-b}{2N}\sum_{k=1}^{N}[f(x_{k+1}) + f(x_k)] \qquad \text{Eq. 2.5}$$

Where (a – b) is the time spacing, and [$f(x_k)$, $f(x_{k+1})$] are the measurements obtained by the oxygen sensor. The data obtained by the sensor is showed in Appendix 1. The table 2.3 shows the percentage yield of the three CaO_2 concentrations. The numerical integration was performed by using 0.25 hours as initial point for the three groups (0.02%, 0.10%, and 0.2%), because the oxygen level from 0.2%-CaO_2/medium proportion was superior to the sensor limits (0.6 mol/m^3) during the first minutes. The percentage yield starting was calculated by dividing the amount of oxygen calculated under the curve (numerical integration) over the theoretical oxygen generation. In this conditions the group 0.02% showed a low fractional yield, which can

be attributed to the space between microparticles, because the low printing density, leading a quick reaction during the first 15 minutes. The other two groups with a CaO_2/Medium proportion of 0.1% and 0.2% had a similar percentage yield of 52.4% and 47.2% respectively. The percentage yield of the groups 0.1% and 0.2% may be correlated to the printing layers of the compound that delayed the contact with water, and the increasing of the alkalinity, because the high CaO_2 density particles increased the pH to 7.27 [18].

Table 2.3. Percentage yield of CaO_2/Medium proportion: 0.02%, 0.10% and 0.20%.

CaO_2 concentration w/V	**Total O_2 generated**	$\boldsymbol{Percent\ yield} = \frac{Mearured\ O_2}{Theoretical\ O_2}\ x\ 100$
0.02%	0.273 mol/m^3	35.5%
0.1%	2.018 mol/m^3	52.4%
0.2%	3.640 mol/m^3	47.2%

The results indicate that calcium peroxide microparticles printed in the range of milligrams may release oxygen for a long time period, which are similar with other studies where used calcium peroxide for different applications [19]. Calcium peroxide has been used for incorporating oxygen to scaffolds using highly hydrophobic materials due to the concern of premature oxygen exhaustion [20]. Further studies are suggested for incorporating calcium peroxide to hydrophilic materials scaffolds, because their high biocompatibility, such as collagen, alginate, and chitosan, that have been applied with successful results in tissue engineering applications [21-23].

2.5 Conclusions

Inkjet printing technology has been used to dispense calcium peroxide microparticles in a controlled mass and pattern in a layer-by-layer fashion. Calcium peroxide is a compound that is capable of generating oxygen for long periods, when is in direct contact with water. Oxygen generated from this compound reach levels above normoxic conditions, and when the compound density is increased, the percentage yield is increased, and exhaustion time is prolonged. Using inkjet printing technology to incorporate oxygen-generating biomaterials to hydrogel scaffolds may improve cells environment in hypoxic conditions.

2.5 REFERENCES

[1] Harrison, Benjamin S., Daniel Eberli, Sang Jin Lee, Anthony Atala, and James J. Yoo. "Oxygen producing biomaterials for tissue regeneration." Biomaterials 28, no. 31 (2007): 4628-4634.

[2] Oh SH, Ward CL, Atala A, Yoo JJ, Harrison BS. Oxygen generating scaffolds for enhancing engineered tissue survival. Biomaterials. 2009;30:757-62.

[3] Xu T, Jin J, Gregory C, Hickman JJ, Boland T. Inkjet printing of viable mammalian cells. Biomaterials. 2005;26:93-9.

[4] Roth EA, Xu T, Das M, Gregory C, Hickman JJ, Boland T. Inkjet printing for high-throughput cell patterning. Biomaterials. 2004;25:3707-15.

[5] Collier WA, Janssen D, Hart AL. Measurement of soluble L-lactate in dairy products using screen-printed sensors in batch mode. Biosens Bioelectron. 1996;11:1041-9.

[6] Hughes TR, Mao M, Jones AR, Burchard J, Marton MJ, Shannon KW, et al. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat Biotechnol. 2001;19:342-7.

[7] Lemmo AV, Rose DJ, Tisone TC. Inkjet dispensing technology: applications in drug discovery. Curr Opin Biotechnol. 1998;9:615-7.

[8] Boland T, Xu T, Damon B, Cui X. Application of inkjet printing to tissue engineering. Biotechnol J. 2006;1:910-7.

[9] Mohebi MM, Evans JR. A drop-on-demand ink-jet printer for combinatorial libraries and functionally graded ceramics. J Comb Chem. 2002;4:267-74.

[10] Boland, Thomas, Xu Tao, Brook J. Damon, Brian Manley, Priya Kesari, Sahil Jalota, and Sarit Bhaduri. "Drop-on-demand printing of cells and materials for designer tissue constructs." Materials Science and Engineering: C 27, no. 3 (2007): 372-376.

[11] Rodríguez-Dévora, Jorge I., Bimeng Zhang, Daniel Reyna, Zhi-dong Shi, and Tao Xu. "High throughput miniature drug-screening platform using bioprinting technology." Biofabrication 4, no. 3 (2012): 035001.

[12] Haselgrove, JOHN C., IRVING M. Shapiro, and SUSAN F. Silverton. "Computer modeling of the oxygen supply and demand of cells of the avian growth cartilage." American Journal of Physiology 265 (1993): C497-C497.

[13] Baker AM, HattonW. Calcium peroxide as a seed coating material for padi rice .1. Requirement for and provision of oxygen. Plant and Soil 1987;99:357–63.

[14] Hanh DN, Rajbhandari BK, Annachhatre AP. Bioremediation of sediments from intensive aquaculture shrimp farms by using calcium peroxide as slow oxygen release agent. Environmental Technology 2005;26:581–9.

[15] Pedraza E, Coronel MM, Fraker CA, Ricordi C, Stabler CL. Preventing hypoxia-induced cell death in beta cells and islets via hydrolytically activated, oxygen-generating biomaterials. P Natl Acad Sci USA. 2012;109:4245-50.

[16] Ma Y, Zhang BT, Zhao LX, Guo GS, Lin JM. Study on the generation mechanism of reactive oxygen species on calcium peroxide by chemiluminescence and UV-visible spectra. Luminescence 2007;22:575–80.

[17] Sinensky MC, Leiser AL, Babich H. Oxidative stress aspects of the cytotoxicity of carbamide peroxide – in-vitro studies. Toxicology Letters 1995;75:101–9.

[18] Northup, Abraham, and Daniel Cassidy. "Calcium peroxide (CaO< sub> 2</sub>) for use in modified Fenton chemistry." Journal of hazardous materials 152, no. 3 (2008): 1164-1170.

[19] Cassidy, Daniel P., and Robert L. Irvine. "Use of calcium peroxide to provide oxygen for contaminant biodegradation in a saturated soil." Journal of hazardous materials 69, no. 1 (1999): 25-39.

[20] Wang, Junping, Yizhou Zhu, Harinder K. Bawa, Geoffrey Ng, Yong Wu, Matthew Libera, H. C. van der Mei, H. J. Busscher, and Xiaojun Yu. "Oxygen-generating nanofiber cell scaffolds with antimicrobial properties." ACS applied materials & interfaces 3, no. 1 (2010): 67-73.

[21] Ma, Lie, Changyou Gao, Zhengwei Mao, Jie Zhou, Jiacong Shen, Xueqing Hu, and Chunmao Han. "Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering." Biomaterials 24, no. 26 (2003): 4833-4841.

[22] Dar, Ayelet, Michal Shachar, Jonathan Leor, and Smadar Cohen. "Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds." Biotechnology and bioengineering 80, no. 3 (2002): 305-312.

[23] Madihally, Sundararajan V., and Howard WT Matthew. "Porous chitosan scaffolds for tissue engineering." Biomaterials 20, no. 12 (1999): 1133-1142.

Chapter 3

Oxygen Generating Scaffolds

3.1 INTRODUCTION

One of the current challenges in tissue engineering is the development of methods to increase oxygen availability for cells within engineered tissues. In the early period of tissue implantation, an inadequate oxygen delivery occurs due to the angiogenesis delay [1], inducing necrosis to cells farther than 200 μm from vasculature [2, 3]. Oxygen generating scaffolds may present a solution to overcome this challenge.

Oxygen concentration gradients are critical for tissue engineering scaffolds, since hypoxia decreases generation of ATP and vital cellular functions are affected, whereas hyperoxia can damage cell membrane and DNA. [4]. Incorporation of calcium peroxide to highly hydrophobic biomaterials such as polydimethylsiloxan (PDMS) and Poly lactic co-glycolic acid (PLGA) scaffolds were studied [5, 6]. However, investigation in hydrophilic materials has not been performed yet.

Alginate hydrogel is a hydrophilic biomaterial that is used for tissue engineering applications, which include cardiac scaffolds and bone scaffolds [7, 8]. In the chapter 3, we found that 0.1% calcium peroxide solution in media generated oxygen levels from 0.6 mol/m^3 to 0.2 mol/m^3 during the first 4 hours, and continued generating oxygen for 30 hours.

In this study alginate hydrogel was used to encapsulate the calcium peroxide microparticles dispensed by the inkjet printing technology in a designed pattern; this technology has been implemented for different biomedical applications, such as, genomics, drug screening, biosensors, and tissue engineering [9-12]. Oxygen supply from scaffolds was evaluated in function of time, using as control, the lowest suitable oxygen concentration for fibroblast cells (0.05 mol/m^3)[13].

3.2 MATERIALS AND METHODS

3.2.1 Oxygen Generating Scaffolds Construction

Oxygen generating ink was prepared with pure ethanol and calcium peroxide powder (Sigma-Aldrich, St. Louis, MO) with a concentration of 1% wt. A modified commercial HP697C (Hewlett-Packard, USA) printer, and a modified HP29 cartridge were used to print the microparticles of calcium peroxide in a controlled pattern. The ink was filtered (40 μm) to avoid clogging issues during printing. The microparticles were printed onto a glass slide with a pattern of 25 dots. 640 printing layers were required to print 10 mg of CaO_2. Five printed samples were dried out overnight at room temperature.

Alginate (Acros Organics, NJ) solution was prepared with a concentration of 2% wt in sterilized phosphate buffered saline (PBS). The five calcium peroxide samples were placed into a 6-well plate, and the alginate solution was poured into each well plate covering the microparticles. The resulting five CaO_2/alginate concentrations (w/v) were: 0.25%, 0.2%, 0.17%, 0.14% and 0.12%. Hence, the alginates to CaO_2 rations included (w/w) 8:1, 10:1, 12:1, 14:1 and 16:1. Calcium chloride (Acros Organics, NJ) (0.25M) was used to promote cross-linking in the alginate chains. Calcium chloride creates a quick cross-link with alginate, leading to non-uniform hydrogel geometries. Therefore, 5 ml of $CaCl_2$ solution (0.25M) was added to the samples using a sprayer nozzle, resulting in a uniform hydrogel. The remaining calcium chloride solution was removed 5 minutes after the application, and pure ethanol was added to each scaffold before to be placed under direct UV light for 30 minutes. The ethanol was removed, and the scaffolds were washed with sterilized PBS. Afterwards, the scaffolds were placed into a chamber at hypoxic settings (0.01 mol/m^3 O_2, 5% CO_2).

3.2.2 Oxygen Release Measurement

Culture medium was purged for 24 hours under hypoxic conditions in a special chamber (BioSpherix, Lacona, NY) at 0.01 mol/m^3 O_2 and 5% CO_2. Subsequently, 5 ml of culture medium consistent of Dulbecco's modified Eagle's medium, 10% FBS, 500 U/mL penicillin, 500 mg/mL streptomycin (Gibco, San Diego, CA), and 100 U/mL of catalase (Sigma-Aldrich, MO, USA) was added to each scaffold; the oxygen levels were measured using an oxygen sensor (OAKTON Instruments, IL, USA) until oxygen background levels were reached.

3.3 RESULTS

Five samples with 10 mg of calcium peroxide microparticles were printed in 25 dots pattern. Each sample was encapsulated with a different concentration of alginate to evaluate the oxygen generating in function of time. Figure 3.1 shows the scaffolds used for this study.

Figure 3.1. Alginate-Hydrogel Scaffolds.

Figure 3.2 shows the scaffold constructed with 8:1 of alginate per CaO_2. The oxygen concentration was maintained in suitable levels for 45 hours. After 45 hours the oxygen concentration in the culture media drops below the inferior limit of suitable oxygen levels for cell culturing. At 72 hours the oxygen concentration reached background levels.

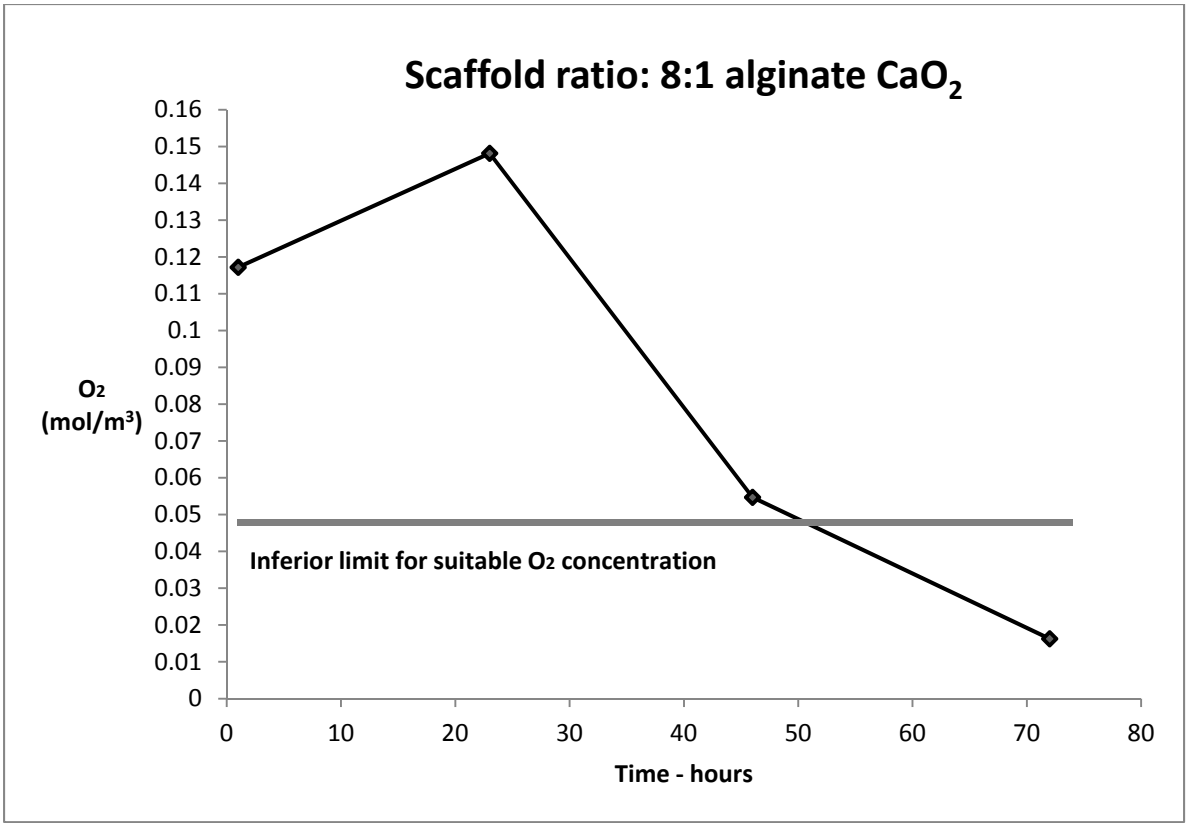

Figure 3.2. Oxygen generating measurements from scaffold ratio: 8:1 alginate CaO_2 (n=1).

Figure 3.3 shows the scaffold with 10:1 alginate-CaO_2 ratios. The maximum oxygen concentration reading was at 22 hours with an oxygen content of 0.13 mol/m^3. After 45 hours the oxygen level was 0.016 mol/m^3 above the control lower limit for cell survival. The culture media reached oxygen background levels at 72 hours.

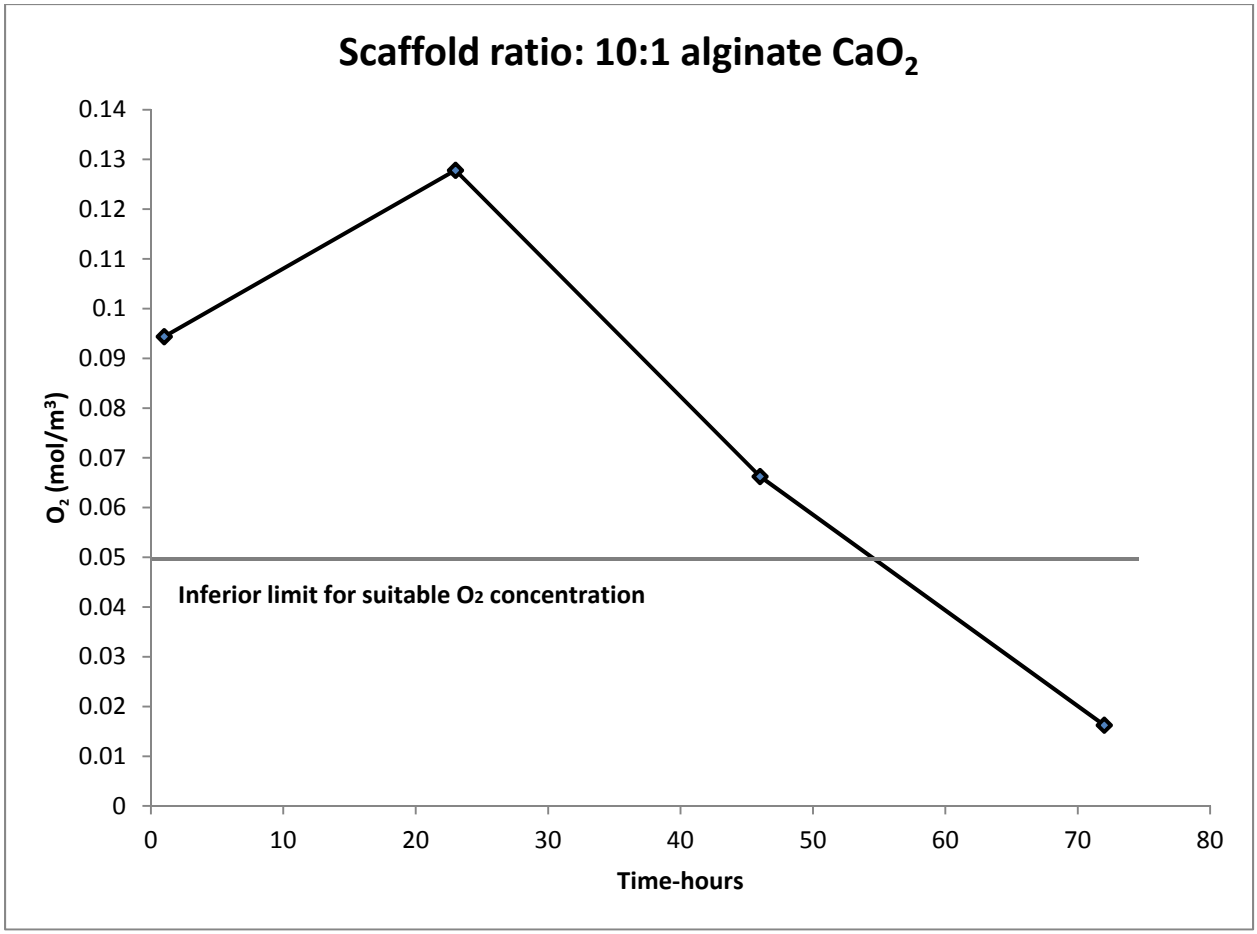

Figure 3.3. Oxygen generating measurements from scaffold ratio: 10:1 alginate CaO_2 (n=1).

Figure 3.4 shows the scaffold with 12:1 alginate-CaO_2 ratios. The maximum oxygen concentration reading was at 22 hours with an oxygen content of 0.098 mol/m^3. Oxygen drops below control levels after 55 hours. At 96 hours the culture medium reaches background oxygen levels.

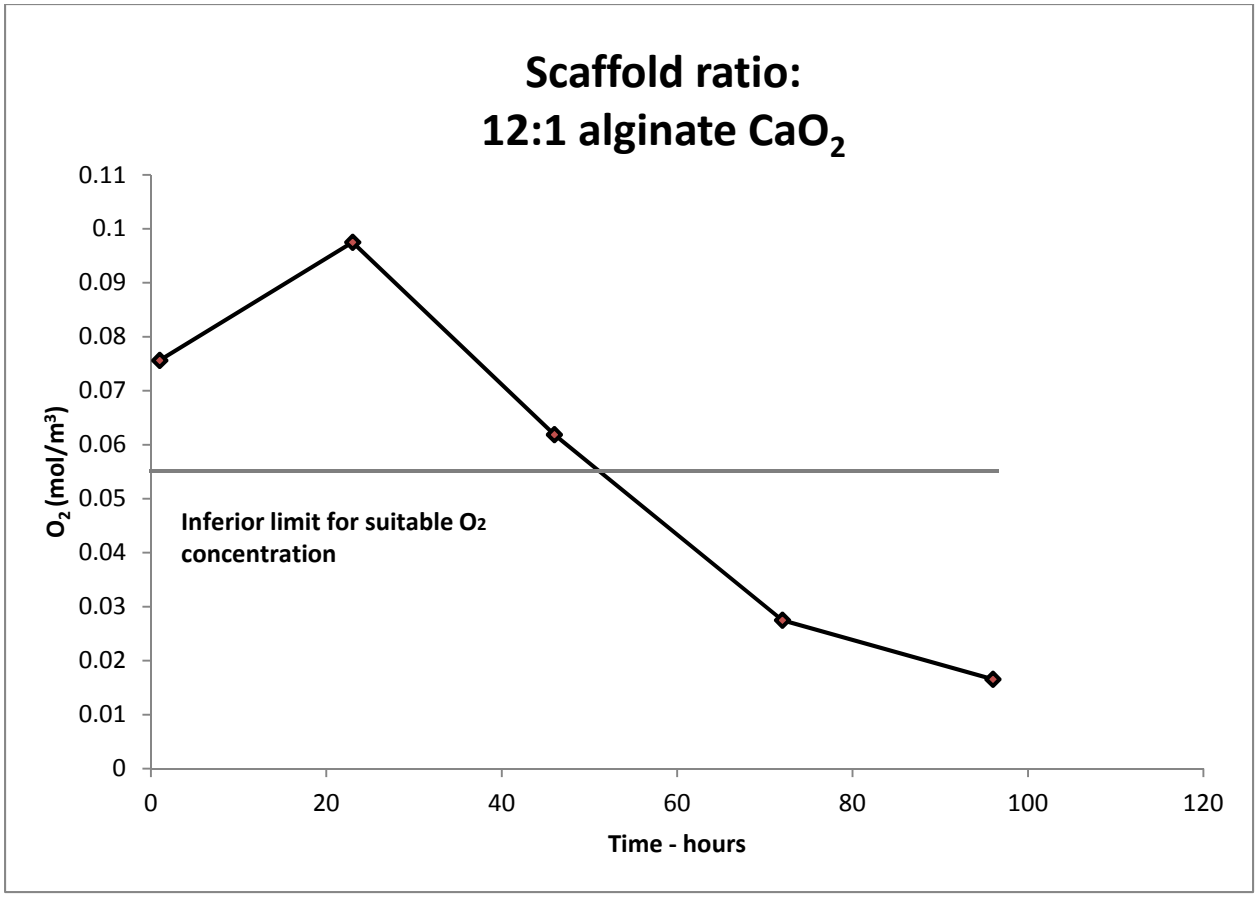

Figure 3.4. Oxygen generating measurements from scaffold ratio: 12:1 alginate CaO_2 (n=1)

Figure 3.5 shows the scaffold with 14:1 alginate-CaO_2 ratios. Oxygen drops below the control limit after 72 hours. The maximum oxygen concentration reading was at 22 hours with a reading of 0.08 mol/m^3. At 120 hours the culture media reached background oxygen concentration levels.

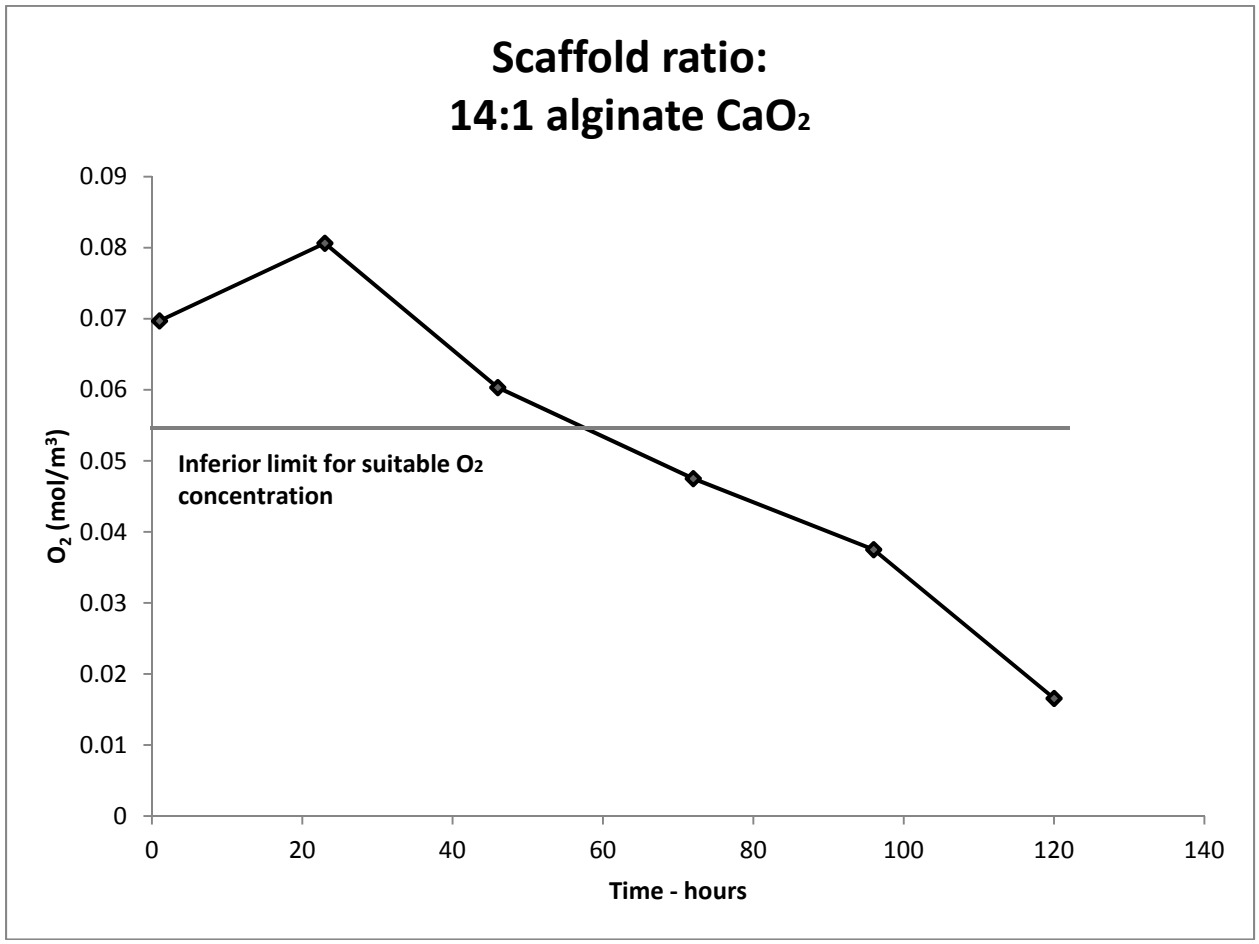

Figure 3.5. Oxygen generating measurements from scaffold ratio: 14:1 alginate CaO_2 (n=1)

Figure 3.6 shows the scaffold with 16:1 alginate CaO_2 ratios. The scaffold maintained oxygen concentration above the lower limit of cell survival of 0.05 mol/m^3 for 120 hours. The maximum oxygen concentration reading was 0.066 mol/m^3 at 22 hours. After 120 hours of scaffold expose to culture medium, the oxygen concentration in the media was 0.052 mol/m^3. After 144 hours the scaffold was degraded.

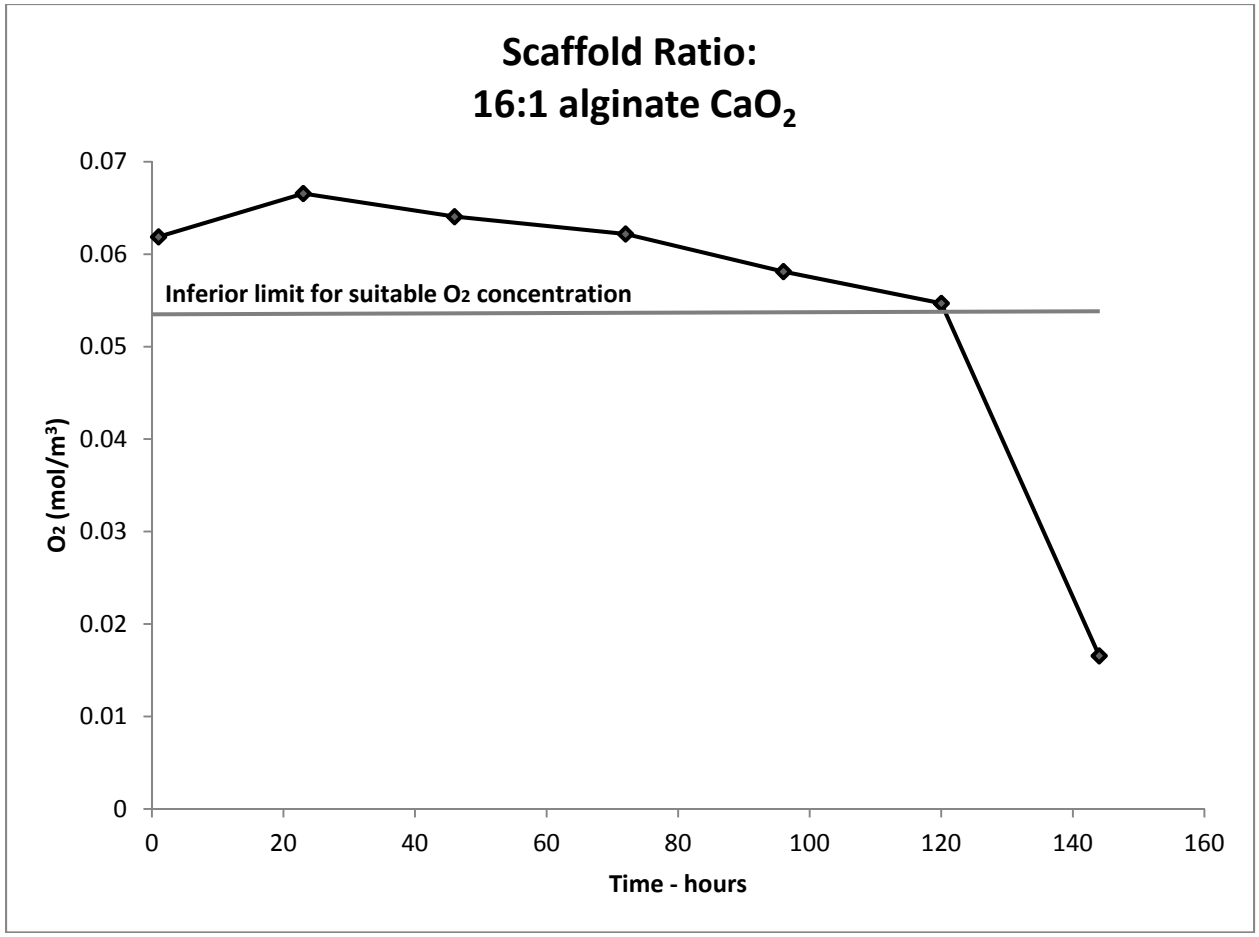

Figure 3.6. Oxygen generating measurements from scaffold ratio: 16:1 alginate CaO_2 (n=1).

3.4 DISCUSSION

Vascularization is required to maintain cell viability within tissue implants [14]. Biomaterials mixed with oxygen generating compounds have been investigated to prolong cell survival under hypoxic environment [5, 6]. However, oxygen gradients are present on the scaffolds, and cell damage may occur [4]. In this study, oxygen generating scaffolds were fabricated by encapsulating oxygen generating particles that were printed in a designed pattern. Oxygen supply was evaluated under hypoxia.

Calcium peroxide was selected as oxygen-generating compound [15, 16], which was encapsulated with alginate hydrogel; oxygen generation was maintained for several days. Calcium peroxide is used in different industries, such as agriculture to release oxygen for long period of time [17]. Alginate is commonly used in tissue engineering applications [18], and drug delivery [19], because its properties, including biocompatibility, hydrolytically degradable, low toxicity, and gelation can be promoted easily by divalent cations, such as Ca^{2+} [20]. Hydrogen peroxide is generated in the first step reaction of calcium peroxide; hence, catalase was added to the culture medium to induce decomposition of residual reactive oxygen species [21-23].

Oxygen generating scaffolds were prepared at five different concentrations of alginate, for encapsulating calcium peroxide particles. The scaffolds were constructed and placed into a chamber at hypoxic settings. After scaffolds were exposure to the culture medium, bubbles were observed on the surface. Figure 3.7 shows the tendency of the oxygen generating in function of the concentration of alginate hydrogel. Scaffolds with the highest relative concentration of alginate showed a prolonged oxygen generating. Alginate degradation was observed when oxygen measurements reached background levels, which it can be attributed to the increasing of the pH inside the scaffold, induced by the presence of H_2O_2 [24].

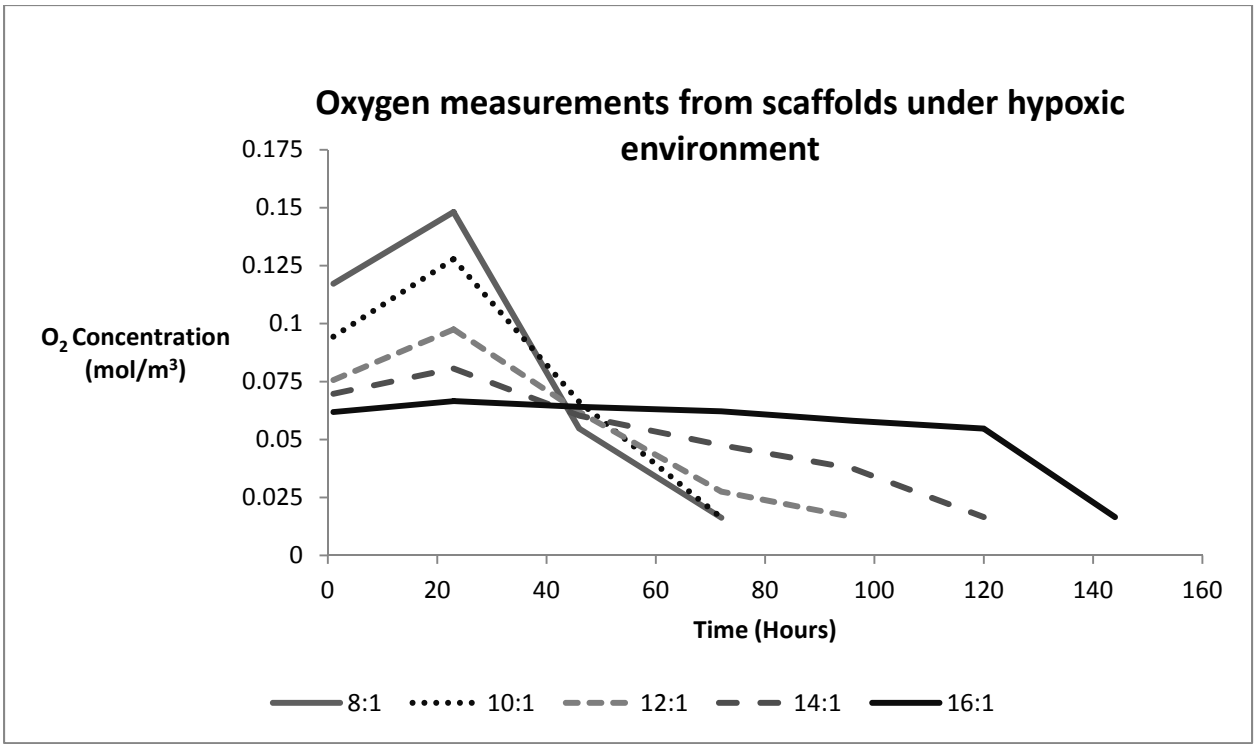

Figure 3.7. Oxygen generating measurements from scaffolds under hypoxic conditions; relative concentrations 8:1, 10:1, 12:1, 14:1 and 16:1

The results indicate that incorporating calcium peroxide into alginate scaffolds can provide oxygen concentrations above 0.05 mol/m^3 for a period of 120 hours. Scaffold containing 16:1 (alginate: CaO_2) had a stable oxygen supply similar to PLGA scaffolds evaluated in previous studies [5]. Table 3.1 shows the reaction efficiency of calcium peroxide encapsulated and non-encapsulated when is in direct contact with culture medium. The CaO_2 reaction data was obtained in previous chapter. CaO_2 exhausted in 30 hours when exposed to culture medium, while CaO_2 encapsulated with alginate hydrogel exhausted in 140 hours. The area under the curve was estimated by numerical integration as described below:

$$\int_a^b f(x)dx \approx \frac{a-b}{2N}\sum_{k=1}^{N}[f(x_{k+1}) + f(x_k)] \qquad \text{Eq. 3.1}$$

The percentage yield was estimated by dividing the area under the curve (mol/m^3) over the total amount of theoretical O_2 that can be generated by the CaO_2/Medium correlation of 0.2%. The data is showed in Appendix 1. The oxygen generated by the scaffolds was compared

to the results obtained in previous chapter, where calcium peroxide particles were exposure directly to medium. The results indicate a higher percent yield in the oxygen generating scaffolds (16:1) than non-encapsulated CaO_2. These were attributed to errors measurements and an overestimation, because the O_2 diffusion into the atmosphere was not accounted for.

Table 3.1. Percentage yield of Scaffold ratio 16:1 (alginate-CaO_2), and CaO_2/medium correlation of 0.2%

Group	**Total O_2 generated**	$\boldsymbol{Percent\ yield} = \frac{Mearured\ O_2}{Theoretical\ O_2}\ x\ 100$
Scaffold ratio 16:1 (alginate-CaO_2)	6.087 mol/m^3	78.96%
CaO_2/Medium 0.2%	3.640 mol/m^3	47.2%

This study indicates that oxygen generating scaffolds fashioned with calcium peroxide microparticles encapsulated with alginate hydrogels can increase oxygen concentrations to suitable cellular conditions when the scaffold is surrounding of a hypoxic environment. This scaffold maintains oxygen levels appropriate for fibroblast cells [9] for a period of 120 hours. Oxygen measurements were performed to the culture media; hence, high oxygen concentration may be present inside the scaffold. These results suggested evaluating this oxygen generating scaffold with fibroblast cells under a hypoxic environment, and asses cell viability for a period of 120 hours.

3.4 CONCLUSIONS

Oxygen generating scaffolds capable of provide suitable oxygen concentrations were constructed. Calcium peroxide microparticles were used as oxygen generating compounds. These microparticles were dispensed by the inkjet printing technology, where position and mass were controlled. Calcium peroxide microparticles provide continuous oxygen generating for a long

period when encapsulated with alginate hydrogels. This study shown that oxygen generating by alginate/CaO_2 scaffolds are able to supply oxygen concentration suitable for cell culturing. This scaffold may represent a significant advance for tissue engineering where oxygen supply limited the creation of large engineered implants.

3.5 REFERENCES

[1] Folkman J, Hochberg M. Self-regulation of growth in three dimensions. J Exp Med. 1973;138:745-53.

[2] Haraguchi Y, Sekine W, Shimizu T, Yamato M, Miyoshi S, Umezawa A, et al. Development of a New Assay System for Evaluating the Permeability of Various Substances Through Three-Dimensional Tissue. Tissue Eng Part C-Me. 2010;16:685-92.

[3] Muschler GE, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am. 2004;86A:1541-58.

[4] Semenza, Gregg L. "Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1." Annual review of cell and developmental biology 15, no. 1 (1999): 551-578.

[5] Oh SH, Ward CL, Atala A, Yoo JJ, Harrison BS. Oxygen generating scaffolds for enhancing engineered tissue survival. Biomaterials. 2009;30:757-62.

[6] Pedraza E, Coronel MM, Fraker CA, Ricordi C, Stabler CL. Preventing hypoxia-induced cell death in beta cells and islets via hydrolytically activated, oxygen-generating biomaterials. P Natl Acad Sci USA. 2012;109:4245-50.

[7] Shachar, Michal, Orna Tsur-Gang, Tal Dvir, Jonathan Leor, and Smadar Cohen. "The effect of immobilized RGD peptide in alginate scaffolds on cardiac tissue engineering." Acta biomaterialia 7, no. 1 (2011): 152-162.

[8] Li, Zhensheng, Hassna R. Ramay, Kip D. Hauch, Demin Xiao, and Miqin Zhang. "Chitosan–alginate hybrid scaffolds for bone tissue engineering." Biomaterials 26, no. 18 (2005): 3919-3928.

[9] Boland T, Xu T, Damon B, Cui X. Application of inkjet printing to tissue engineering. Biotechnol J. 2006;1:910-7.

[10] Collier WA, Janssen D, Hart AL. Measurement of soluble L-lactate in dairy products using screen-printed sensors in batch mode. Biosens Bioelectron. 1996;11:1041-9.

[11] Hughes TR, Mao M, Jones AR, Burchard J, Marton MJ, Shannon KW, et al. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat Biotechnol. 2001;19:342-7.

[12] Lemmo AV, Rose DJ, Tisone TC. Inkjet dispensing technology: applications in drug discovery. Curr Opin Biotechnol. 1998;9:615-7.

[13] Taylor GW, Kondig JP, Nagle SC, Jr., Higuchi K. Growth and metabolism of L cells in a chemically defined medium in a controlled environment culture system. I. Effects of O_2 tension on L-cell cultures. Appl Microbiol. 1971;21:928-33.

[14] Smith, Molly K., Martin C. Peters, Thomas P. Richardson, Jessica C. Garbern, and David J. Mooney. "Locally enhanced angiogenesis promotes transplanted cell survival." Tissue engineering 10, no. 1-2 (2004): 63-71.

[15] Northup A, Cassidy D. Calcium peroxide (CaO_2) for use in modified Fenton chemistry. J Hazard Mater. 2008;152:1164-70.

[16] Waite AJ, Bonner JS, Autenrieth R. Kinetics and stoichiometry of oxygen release from solid peroxides. Environ Eng Sci. 1999;16:187-99.

[17] Baker AM, HattonW. Calcium peroxide as a seed coating material for padi rice .1. Requirement for and provision of oxygen. Plant and Soil 1987;99:357–63.

[18] Sapir, Yulia, Olga Kryukov, and Smadar Cohen. "Integration of multiple cell-matrix interactions into alginate scaffolds for promoting cardiac tissue regeneration." Biomaterials 32, no. 7 (2011): 1838-1847.

[19] Gombotz, Wayne R., and Siow Fong Wee. "Protein release from alginate matrices." Advanced drug delivery reviews 64 (2012): 194-205.

[20] Smidsrød, Olav. "Alginate as immobilization matrix for cells." Trends in biotechnology 8 (1990): 71-78.

[21] Chelikani P, Fita I, Loewen PC. Diversity of structures and properties among catalases. Cell Mol Life Sci. 2004;61:192-208.

[22] Raducan A, Cantemir AR, Puiu M, Oancea D. Kinetics of hydrogen peroxide decomposition by catalase: hydroxylic solvent effects. Bioproc Biosyst Eng. 2012;35:1523-30.

[23] Schmidtke T, White D, Woolard C. Oxygen release kinetics from solid phase oxygen in Arctic Alaska. J Hazard Mater. 1999;64:157-65.

[24] Bouhadir, Kamal H., Kuen Yong Lee, Eben Alsberg, Kelly L. Damm, Kenneth W. Anderson, and David J. Mooney. "Degradation of partially oxidized alginate and its potential application for tissue engineering." Biotechnology progress 17, no. 5 (2001): 945-950.

Chapter 4

Oxygen Generating Scaffolds to Enhance Cell Viability under Hypoxic Environment

4.1 INTRODUCTION

Creation of large engineered tissues is still a challenge for tissue engineering because the slow rates of angiogenesis [1]. To overcome this limitation, different methods are proposed, all aiming at providing a suitable oxygen supply to engineered tissues. These include growth factors, synthetic oxygen carries, and oxygen generating biomaterials [2-4]. Oxygenated scaffolds were created in previous studies mixing hydrophobic materials with oxygen generating compounds for improving cell environment [4, 5]. However studies with hydrophilic materials and hydrogels have not been performed yet. In addition, improvements of oxygen generating scaffolds that control the oxygen generated with better spatial resolution are warranted, because the existing materials merely generate oxygen gradients which can induce hypoxia affecting the ATP generation from cells, or hyperoxia that can damage cell membrane [6]. Inkjet printing may be in particular advantage, dispensing biomaterials and/or microparticles in a controlled pattern [7], this technology can be applied to deposit biomaterials with micro-gram precision. Calcium peroxide (CaO_2) when is in contact with water decomposes in hydrogen peroxide. Oxygen is generated in a second reaction [8], which requires a catalyst to accelerate the decomposition and prevent cell damage [9, 10, 11]. Naturally derived hydrogels are used in tissue engineering as scaffolds and drug delivery vehicles. Alginate hydrogel, in particular, is a naturally derived hydrophilic polymer, which is biocompatible, and gelation is achieved with divalent cations such as Ca^{++} under mild conditions [12]. Alginate hydrogels are used in cardiac tissue engineering application amongst others [13]. In this study oxygen generating scaffolds were constructed to improve cell viability under hypoxic conditions. CaO_2 microparticles were printed in a controlled pattern and density, encapsulated with alginate hydrogel to provide a continuous oxygen supply.

Fibroblast L-Cells were selected as a cell model, and previous studies demonstrated that mitochondrial activity of this cell line is optimal in oxygen levels of 0.05 mol/m^3 to 0.13 mol/m^3[15], which makes this cell line ideal to study the effect of hypoxia and oxygen generating from tissue engineering scaffolds.

4.2 MATERIALS AND METHODS

4.2.1 MTS assay under Normoxic and Hypoxic conditions

Fibroblast L-cells were donated by Dr. Armando Varela coordinator of I Lab/Research at the University of Texas at El Paso. L-cells were cultured for 3 days in standards conditions (0.18 mol/m^3 O_2, 37 ºC, 5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) augmented with 10% Fetal Bovine Serum (FBS), and 5% of penicillin, all obtained from Gibco (San Diego, CA). Cultured cells were trypsinized and divided in two equal batches. One batch was placed in a lab culture hood, the other in a hypoxic chamber. Five different cell concentrations were prepared for each batch: $1x10^5$ cells/mL, $2x10^5$ cells/mL, $3x10^5$ cells/mL, and $4x10^5$ cells/mL.

Scaffolds were formed by physically cross-linking of alginate chains (Acros Organics, NJ) dissolved in sterilized phosphate buffered saline (PBS) (2% w/v) with calcium chloride (Acros Organics, NJ) (0.25M). The gels were sterilized by washing thoroughly with ethanol and placing under UV light for 30 minutes. Scaffolds were washed with sterile PBS prior to be transferred to well-plates. CellTiter 96® aqueous one solution reagent (MTS) obtained from Promega (Madison, WI) was used to evaluate the mitochondrial activity from L-cells under hypoxic and normoxic environments. L-cells were seeded onto alginate scaffolds at different densities: $1x10^5$cells/mL, $2x10^5$ cells/mL, $3x10^5$ cells/mL, and $4x10^5$ cells/mL. MTS assays were performed 1 hour after cells seeding. Subsequently, 20µl of MTS was added per 100 µl of media in each well. Plates were incubated for 2 hours at 37 ºC, the DMEM/MTS solutions were transferred into new wells to avoid hydrogel interference, and the absorbance was read at 490 nm using an automated plate reader (BioTek, Winooski, VT). As various conditions (media, serum,

pH, light exposure, presence of other chemicals, light exposure) can affect the background absorbance, 'no cell' controls were included for background adjustment.

4.2.2 Oxygen generating scaffolds construction

Oxygen generating scaffolds were fabricated by encapsulating oxygen generating particles in alginate. 10 mg of calcium peroxide microparticles obtained from Sigma-Aldrich (St. Louis, MO) were suspended in 200-proof ethanol, filtered, pipetted into a black inkjet cartridge (HP29) and printed layer-by-layer with a modified inkjet printer HP692C, forming a 25 dots pattern, which was designed using Microsoft PowerPoint. The black cartridge was emptied and rinsed thoroughly with ethanol prior to introduce the CaO_2 suspension. The printed patterns were encapsulated with a 2% alginate (Acros Organics, NJ) at a solution in PBS. Then, 0.25M calcium chloride solution in DI water (Acros Organics, NJ) was sprayed onto the alginate to obtain a scaffold. Alginate to calcium peroxide ratio was 16:1. Control scaffolds without CaO_2 were fabricated with only alginate. Oxygen generating scaffolds, and control scaffolds were washed with ethanol and exposed to UV light radiation for 30 minutes. Scaffolds were washed with sterilized PBS prior transferred to well-plates.

4.2.3 Oxygen Generating Scaffolds Evaluation

L-cells were seeded onto each scaffold. 100 U/ml of catalase (Sigma-Aldrich, St. Louis, MO) was added to the media to react with hydrogen peroxide byproducts. Scaffolds cultured at normoxic (0.18 mol/m^3 O_2, 5% CO_2, 37 ºC) were positive control. The remaining scaffolds were cultured at hypoxic conditions (0.01 mol/m^3 O_2, 5% CO_2, 37 ºC) using a specially designed culture system (BioSpherix, Lacona, NY), where negative control scaffolds contained no CaO_2; the other scaffolds containing the 16:1 ratio of alginate to CaO_2 were labeled as oxygen generating (OG) scaffolds. The mitochondrial activity was evaluated every day for each control and the OG scaffolds by the MTS assay.

Cell viability of the L-cells was evaluated after 120 hours with LIVE/DEAD assay (LIVE/DEAD viability/Cytotoxicity kit) bought from Invitrogen. Culture media was removed for

each scaffold group and the cells washed with Dulbecco's phosphate saline solution (PBS) (Sigma). A solution was prepared containing a ratio of 4µL of Ethidium homodimer-1 (EthD-1) - 1µL of calcein per 2 mL of PBS. The LIVE/DEAD assay solution was poured onto each scaffold until the liquid covered 1 mm level above the scaffold surface. The samples were incubated at normoxic environment (0.18 mol/m^3 O_2, 5% CO_2, 37 ºC) for 30 minutes. The LIVE/DEAD assay solution was removed, and 10% buffer formalin solution was added to fix the cells to the scaffolds. After 30 minutes with 10% buffer formalin solution in the dark and room temperature, the scaffolds were transferred to a petri-dish with the top surface of the scaffold inverted in the base of the plate, for observing the cells using a confocal microscope Nikon-D Eclipse (Nikon Instruments, Melville, NY).

4.3 RESULTS

The results of the mitochondrial activity evaluation of the L-cells seeded onto alginate hydrogel scaffolds are represented in the figure 4.1, which show the absorbance values as function of the cell density per mL under normoxic and hypoxic environments. These results show that oxygen concentration affects the absorbance values obtaining different results at normoxia in comparison with hypoxia using same cell density. The absorbance values at normoxia were approximately 1.75 times higher than cells evaluated at hypoxia. The trendline equations obtained to predict cell density with absorbance values at normoxia and hypoxia conditions are showed below:

$$Cells\ per\ cm^2\ (Normoxia) = \frac{Absorbance\ Value}{1x10^{-6}} \qquad \text{Eq. 4.1}$$

$$Cells\ per\ cm^2\ (Hypoxia) = \frac{Absorbance\ Value}{6x10^{-7}} \qquad \text{Eq. 4.2}$$

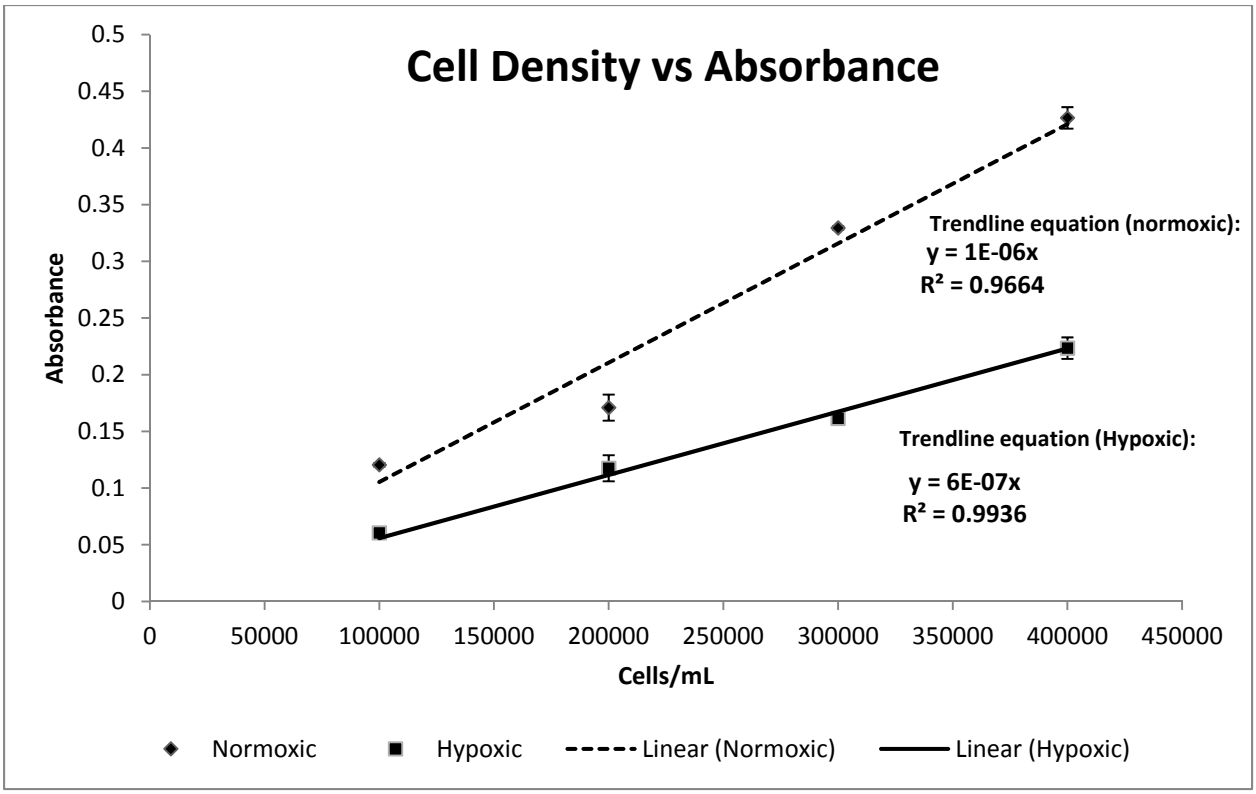

Figure 4.1. Absorbance values with different cells density at normoxic (0.18 mol) and hypoxic (0.01 mol) conditions. Cells were seeded onto alginate-hydrogel scaffolds. Absorbance values were obtained by MTS assay. Trendline equations allow cell/absorbance calculation.

The figure 4.3 shows the alginate-hydrogel scaffold surface, where porosity can be seen that was generated by the spraying calcium chloride solution. In the figure 4.2 plots of the L-cells mitochondrial activity that was evaluated onto the oxygen generating (OG) scaffolds and positive (non-CaO_2, normoxia) and negative controls (non-CaO_2, hypoxia) are shown. Figure 4.4 shows the absorbance values converted to number of cells per mL using the equations 4.1 and 4.2 for normoxia and hypoxia environments respectively. The cell numbers of the OG Scaffolds were statistical different with respect to negative control ($P < 0.05$). The cell number decreased with time in all the groups because the low protein adsorption onto the alginate-hydrogel. Figure 4.5 shows the normalized cell number versus time, where the cell number in the negative control scaffold (Non-CaO_2, hypoxia) decreases 79% after 120 hours of culturing. The positive control

scaffold (non-CaO_2, normoxia), and OG scaffold had 68% and 63% of cells remaining after 120 hours of culturing.

The cell viability at 120 hours is shown in figures from 4.5 to 4.7. These images correlates well with the results obtained from the MTS assay. Where in the positive control incubated at normoxic conditions showed the highest cell density attached to the surface of the scaffolds; the oxygen generating scaffold showed less cell density, and the negative control showed the least cells attached to the scaffold. The figure 4.6 also shows that the cells primarily grew in the porous areas that were created by the spraying.

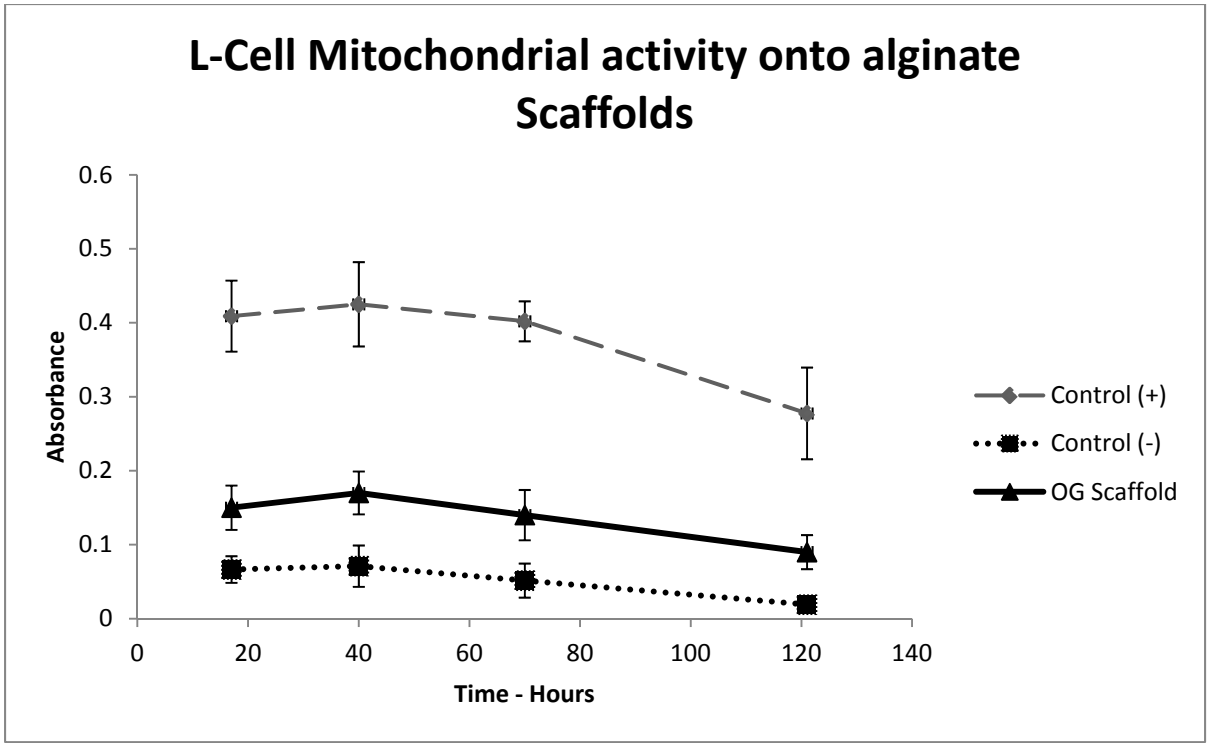

Figure 4.2. L-cells mitochondrial activity onto alginate scaffolds. Oxygenized scaffolds (OG) had a higher absorbance in comparison with negative control at hypoxic conditions of 0.01 mol/m^3. (n=3) $P < 0.05$ between OG Scaffold and Control (-). $P<0.05$ between OG Scaffold and Control (+).

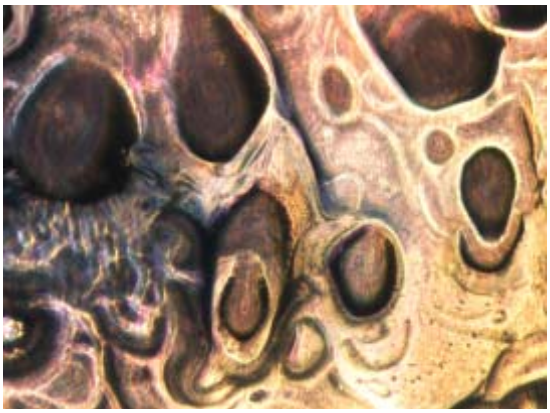

Figure 4.3. Alginate-hydrogel Scaffold Surface

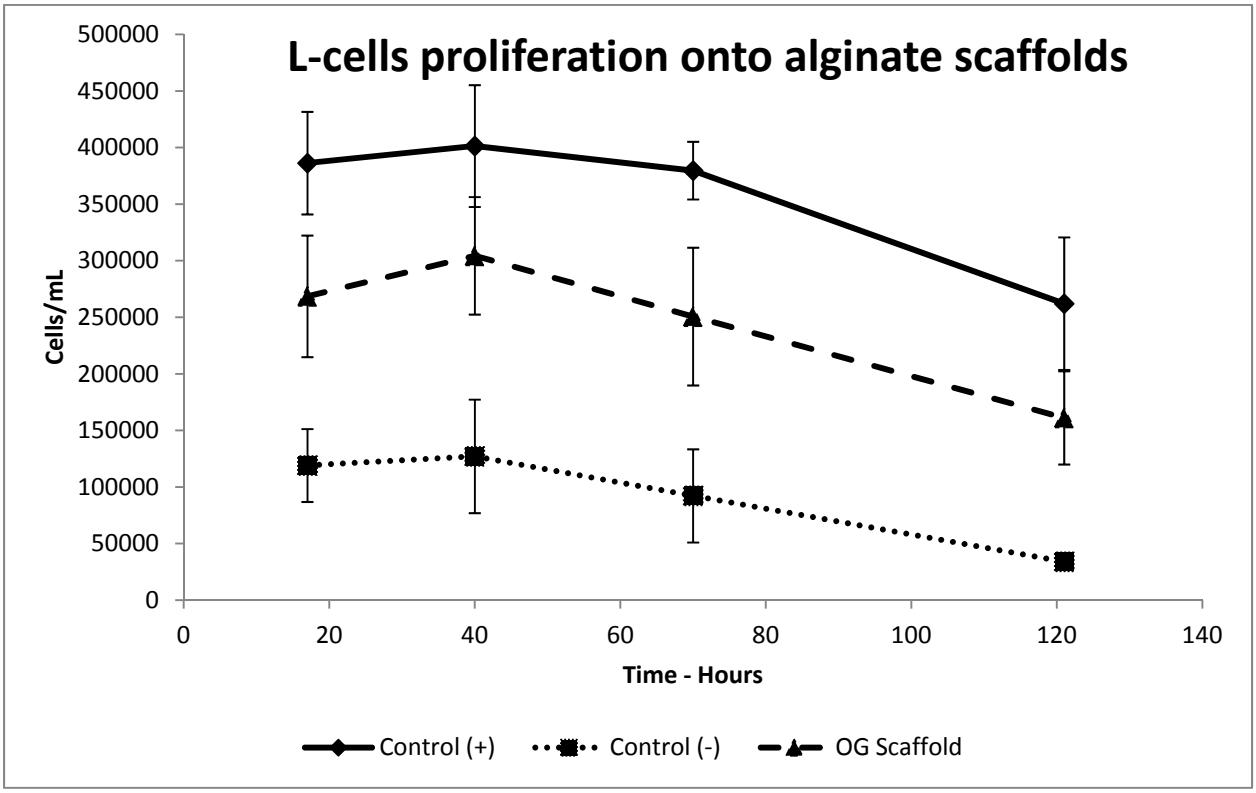

Figure 4.4. L-cells mitochondrial activity of cells grown on alginate scaffolds. Oxygenized scaffolds (OG) Control (-) Control (+) are shown.

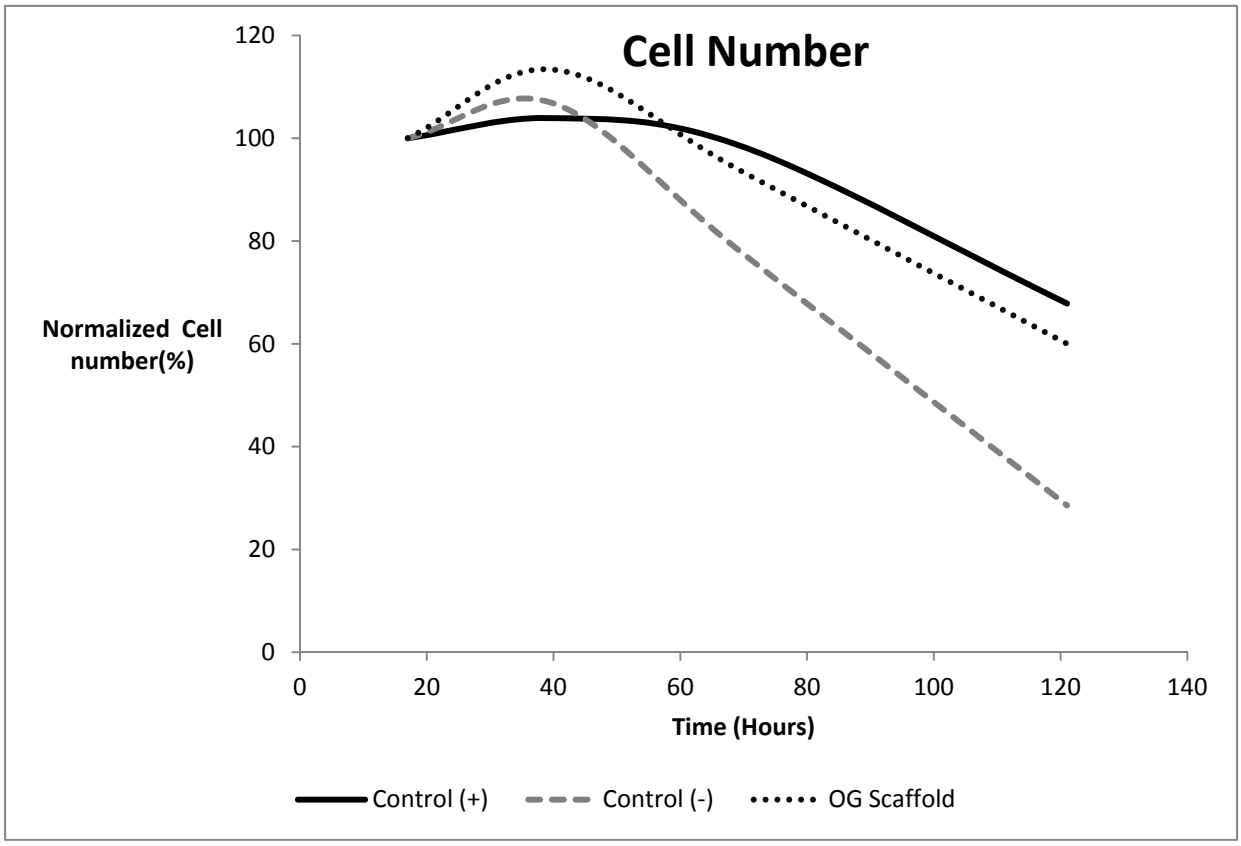

Figure 4.5. Fibroblast cells number over time. Oxygenized scaffolds (OG), control (-), and control (+) are shown.

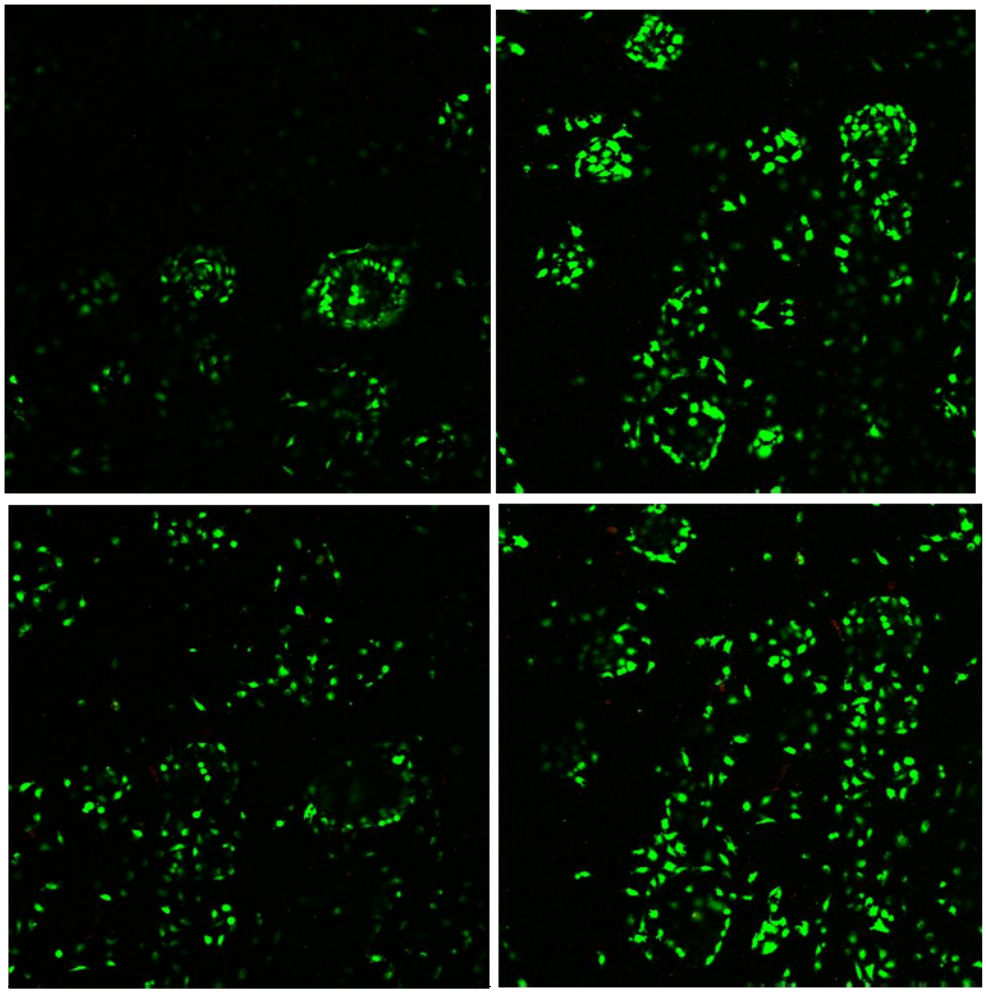

Figure 4.6. L-cells cultured on positive control (+) scaffold. Live/dead assay at 125 hours incubated at normoxic conditions (0.18 mol/m^3, 5% CO_2, 37 ºC).

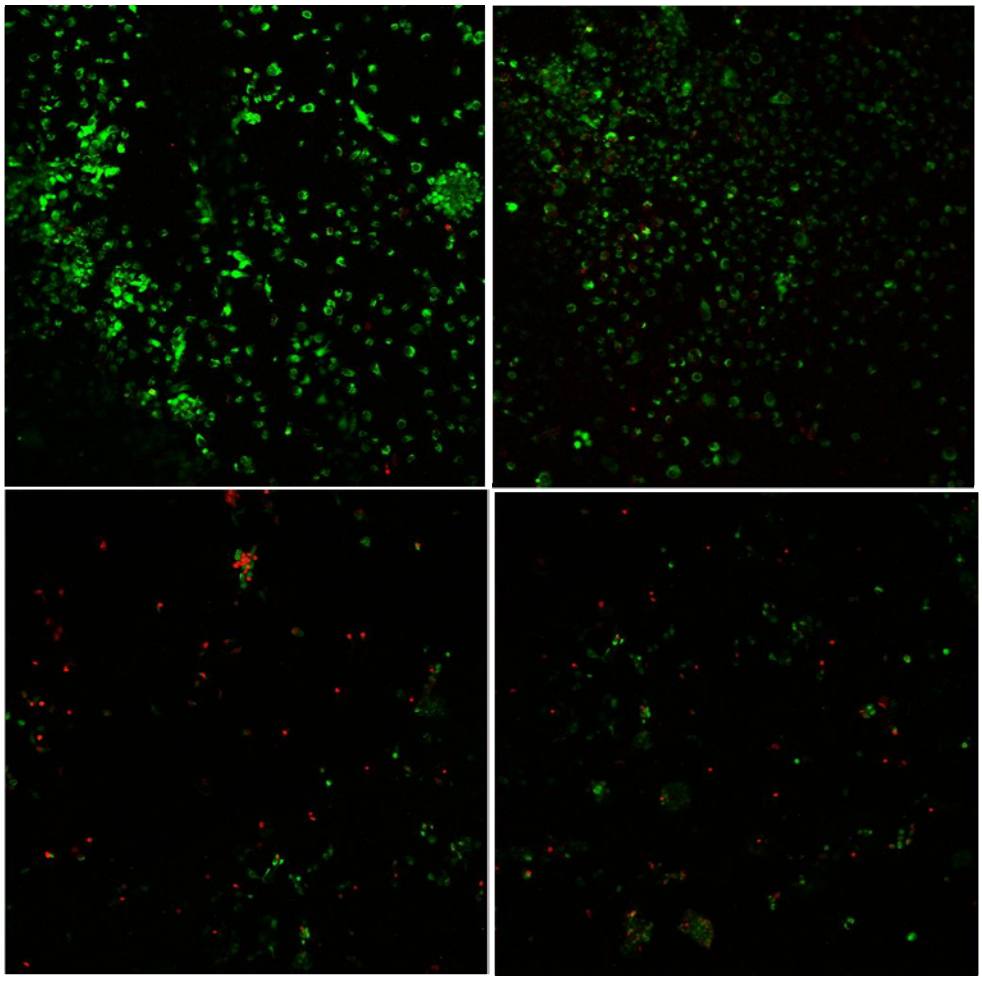

Figure 4.7. L-Cells cultured onto negative control (-) scaffold. Live/Dead Assay at 120 hours incubated under hypoxic conditions (0.01 mol/m^3, 5% CO_2, 37 ºC).

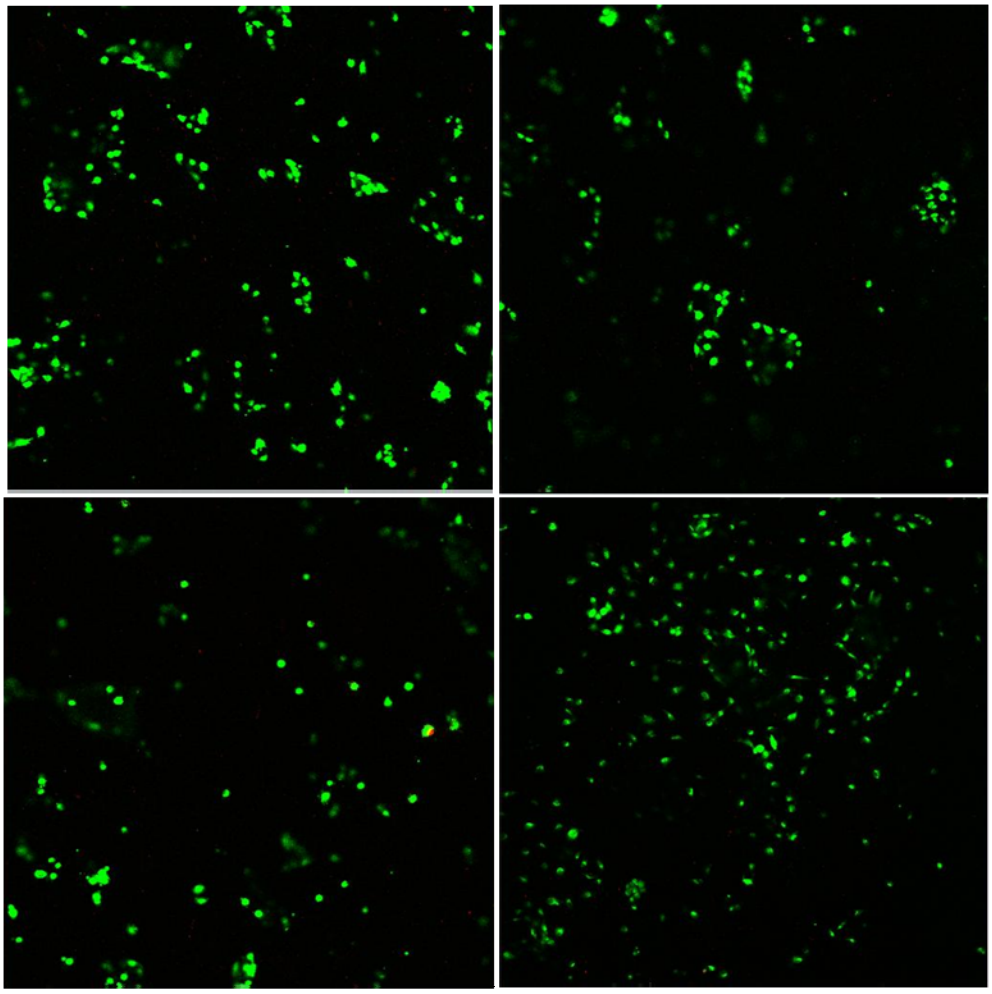

Figure 4.8. L-cells cultured onto oxygenized scaffolds (OG). Live/Dead Assay at 120 hours of incubation at hypoxic conditions (0.01 mol/m^3, 5% CO_2, 37 ºC)

4.4 DISCUSSION

Oxygen supply to cells is a critical factor to consider for engineering large tissues. Because of oxygen diffuses between 100 to 200 μm from blood vessels, only thin tissues are engineered to date [15, 16]. Different strategies are proposed to stimulate vascularization post-implantation in thick tissues, such as angiogenic growth factors (VEFG) [17], and synthetic oxygen carriers (perflurocarbons) [18]. However, angiogenic response occurs at a low rate of 42 μm per hour [19]. Hence, the size of engineered tissues constraint is due to necrosis occurs when is exceeded diffusion limits [20].

In the present study, oxygen generating scaffolds were fabricated by printing calcium peroxide microparticles (< 40 μm) using the inkjet printing technology [21, 22], encapsulating them with alginate hydrogels for providing suitable oxygen supply to cells exposed at hypoxic conditions. Calcium peroxide decomposes via hydration in H_2O_2, and the oxygen is generated in a second step reaction [8]. Catalase was added to the culture medium to ensure decomposition of residual reactive oxygen species [9, 10, 11], avoiding detrimental effect to cells. The role of the alginate-hydrogel was to decrease the timing reaction of CaO_2 and providing a long-term oxygen supply to cells. Scaffolds fabricated with 16:1 alginate to CaO_2 were evaluated under hypoxic incubation for their ability to steadily supply oxygen to cells over time [6, 24]. The results on chapter 3 showed that these oxygen generating scaffolds maintain steady oxygen levels of 0.06 mol/m^3, which is suitable for fibroblast cell culturing [14]. One concern of using calcium peroxide as oxygen generating compound is the presence of hydroxide ions that can change the pH in culture medium. Nevertheless, the pH levels stayed between 6.9 and 7.3 in the culture media over the 120 hours of experiment. Further studies are required to evaluate the pH inside the scaffold.

Mitochondrial activity of fibroblast cells seeded onto scaffolds was evaluated for a period of 120 hours by using MTS assay. The absorbance values obtained from MTS assay were converted to number of cells per mL by using the equations 4.1 and 4.2 obtained from normalizing the absorbance values at normoxic and hypoxic environments. Cell proliferation was

not observed on the scaffolds, because the poor protein absorption onto alginate hydrogels [25]. Alginate hydrogels were modified in previous studies with carbohydrate specific binding proteins, such as lectins to improve properties of cell adhesion and proliferation [26]. Future work will be focusing on modifying alginate to induce cell proliferation [27]. Positive control scaffold and OG scaffold showed higher normalized cell number of 68% and 60% respectively than negative control scaffold with 21% after 120 hours of incubation; most likely due to the lack of oxygen. The metabolic rate in OG scaffolds was higher than negative control scaffolds after 120 hours of hypoxic incubation because the oxygen supply of scaffolds. Cell viability among positive control scaffolds and OG scaffolds was also higher than negative controls in live/dead assays. However, cells grown on positive control scaffolds showed different morphology, they had a larger shape and more intensive fluorescence, indicating higher metabolism than other scaffolds. This suggests that these oxygen-generating scaffolds, while creating a continuous oxygen supply and improving cell viability under hypoxic incubation, are still far from recapitulating the normoxic environment needed for normal cell proliferation and differentiation. However, the OG scaffolds did improve cell viability, and this may be key for building large constructs of engineered tissues. One hypothesis would be that if one can maintain viability for an extended time, neovascularization will slowly restore normoxic conditions in implanted tissues. Vascularization into tissue scaffolds occur approximately 42 μm per hour [28], therefore alginate-hydrogel scaffolds that maintain cell viability for 120 hours would allow implanting a 5 mm thick of tissue, which would be an improvement of at least a factor of 5 over current technology.

Hydrophilic oxygen generating scaffolds may be further tuned by modifying their chemical properties [29] or incorporating others naturally polymers, such as chitosan and collagen [30, 31, 32], for improving cell attachment and proliferation. Future work will explore in vivo responses to these scaffolds.

4.5 CONCLUSIONS

The present study shows the effect of the oxygen generating scaffolds for enhancing cell viability under hypoxic environment *in vitro*. Calcium peroxide particles dispensed by inkjet printers were used as oxygen generating compound, which provided extended oxygen supply when encapsulated with alginate hydrogels. These scaffolds released oxygen into the culturing media improving viability under hypoxia. The use of these biomaterials to create scaffolds may maintain viability for a large period of time, and potentially allowing better vascularization post-implantation. These scaffolds provide an advance to overcome current challenges in tissue engineering, where implantation of large engineered tissues is limited by the oxygen diffusion.

4.5 REFERENCES

[1] Folkman J, Hochberg M. Self-regulation of growth in three dimensions. J Exp Med. 1973;138:745-53.

[2] Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. Nat Biotechnol. 2001;19:1029-34.

[3] Kimelman-Bleich N, Pelled G, Sheyn D, Kallai I, Zilberman Y, Mizrahi O, et al. The use of a synthetic oxygen carrier-enriched hydrogel to enhance mesenchymal stem cell-based bone formation in vivo. Biomaterials. 2009;30:4639-48.

[4] Oh SH, Ward CL, Atala A, Yoo JJ, Harrison BS. Oxygen generating scaffolds for enhancing engineered tissue survival. Biomaterials. 2009;30:757-62.

[5] Pedraza E, Coronel MM, Fraker CA, Ricordi C, Stabler CL. Preventing hypoxia-induced cell death in beta cells and islets via hydrolytically activated, oxygen-generating biomaterials. P Natl Acad Sci USA. 2012;109:4245-50.

[6] Semenza, Gregg L. "Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1." Annual review of cell and developmental biology 15, no. 1 (1999): 551-578.

[7] Boland T, Xu T, Damon B, Cui X. Application of inkjet printing to tissue engineering. Biotechnol J. 2006;1:910-7.

[8] Waite AJ, Bonner JS, Autenrieth R. Kinetics and stoichiometry of oxygen release from solid peroxides. Environ Eng Sci. 1999;16:187-99.

[9] Chelikani P, Fita I, Loewen PC. Diversity of structures and properties among catalases. Cell Mol Life Sci. 2004;61:192-208.

[10] Raducan A, Cantemir AR, Puiu M, Oancea D. Kinetics of hydrogen peroxide decomposition by catalase: hydroxylic solvent effects. Bioproc Biosyst Eng. 2012;35:1523-30.

[11] Schmidtke T, White D, Woolard C. Oxygen release kinetics from solid phase oxygen in Arctic Alaska. J Hazard Mater. 1999;64:157-65.

[12] Smidsrød, Olav. "Alginate as immobilization matrix for cells." Trends in biotechnology 8 (1990): 71-78.

[13] Shachar, Michal, Orna Tsur-Gang, Tal Dvir, Jonathan Leor, and Smadar Cohen. "The effect of immobilized RGD peptide in alginate scaffolds on cardiac tissue engineering." Acta biomaterialia 7, no. 1 (2011): 152-162.

[14] Taylor GW, Kondig JP, Nagle SC, Jr., Higuchi K. Growth and metabolism of L cells in a chemically defined medium in a controlled environment culture system. I. Effects of O_2 tension on L-cell cultures. Appl Microbiol. 1971;21:928-33.

[15] Carmeliet, Peter, and Rakesh K. Jain. "Angiogenesis in cancer and other diseases." nature 407, no. 6801 (2000): 249-257.

[16] Kannan, Ruben Y., Henryk J. Salacinski, Kevin Sales, Peter Butler, and Alexander M. Seifalian. "The roles of tissue engineering and vascularisation in the development of micro-vascular networks: a review." Biomaterials 26, no. 14 (2005): 1857-1875.

[17] Ahrendt G, Chickering DE, Ranieri JP. Angiogenic growth factors: A review for tissue engineering. Tissue Eng. 1998;4:117-30.

[18] Ahrendt G, Chickering DE, Ranieri JP. Angiogenic growth factors: A review for tissue engineering. Tissue Eng. 1998;4:117-30.

[19] Smith MK, Peters MC, Richardson TP, Garbern JC, Mooney DJ. Locally enhanced angiogenesis promotes transplanted cell survival. Tissue Eng. 2004;10:63-71.

[20] Lewis, Miranda C., Ben D. MacArthur, Jos Malda, Graeme Pettet, and Colin P. Please. "Heterogeneous proliferation within engineered cartilaginous tissue: the role of oxygen tension." Biotechnology and bioengineering 91, no. 5 (2005): 607-615.

[21] Xu, Tao, Joyce Jin, Cassie Gregory, James J. Hickman, and Thomas Boland. "Inkjet printing of viable mammalian cells." Biomaterials 26, no. 1 (2005): 93-99.

[22] Roth, Elisabeth A., Tao Xu, Mainak Das, Cassie Gregory, Jay J. Hickman, and Thomas Boland. "Inkjet printing for high-throughput cell patterning." Biomaterials 25, no. 17 (2004): 3707-3715.

[23] Wilson WC, Boland T. Cell and organ printing 1: Protein and cell printers. Anat Rec Part A. 2003;272A:491-6.

[24] Jamieson, Dana, Britton Chance, Enrique Cadenas, and Alberto Boveris. "The relation of free radical production to hyperoxia." Annual review of physiology 48, no. 1 (1986): 703-719.

[25] Smetana Jr, Karel. "Cell biology of hydrogels." Biomaterials 14, no. 14 (1993): 1046-1050.

[26] Sultzbaugh, K. J., and T. J. Speaker. "A method to attach lectins to the surface of spermine alginate microcapsules based on the avidin biotin interaction." Journal of microencapsulation 13, no. 4 (1996): 363-376.

[27] Yu, Jiashing, Yiping Gu, Kim T. Du, Shirley Mihardja, Richard E. Sievers, and Randall J. Lee. "The effect of injected RGD modified alginate on angiogenesis and left ventricular function in a chronic rat infarct model." Biomaterials 30, no. 5 (2009): 751-756.

[28] Cao Y, Mitchell G, Messina A, Price L, Thompson E, Penington A, et al. The influence of architecture on degradation and tissue ingrowth into three-dimensional poly(lactic-co-glycolic acid) scaffolds in vitro and in vivo. Biomaterials. 2006;27:2854-64.

[29] Shachar, Michal, Orna Tsur-Gang, Tal Dvir, Jonathan Leor, and Smadar Cohen. "The effect of immobilized RGD peptide in alginate scaffolds on cardiac tissue engineering." Acta biomaterialia 7, no. 1 (2011): 152-162.

[30] Shao, Xinxin, and Christopher J. Hunter. "Developing an alginate/chitosan hybrid fiber scaffold for annulus fibrosus cells." Journal of Biomedical Materials Research Part A 82, no. 3 (2007): 701-710.

[31] Li, Zhensheng, Hassna R. Ramay, Kip D. Hauch, Demin Xiao, and Miqin Zhang. "Chitosan–alginate hybrid scaffolds for bone tissue engineering." Biomaterials 26, no. 18 (2005): 3919-3928

[32] De Chalain, Tristan, John H. Phillips, and Aleksander Hinek. "Bioengineering of elastic cartilage with aggregated porcine and human auricular chondrocytes and hydrogels containing alginate, collagen, and κ-elastin." Journal of biomedical materials research 44, no. 3 (1999): 280-288

Chapter 5

Conclusions

This study was focused on fabricating oxygen generating scaffolds with applications in tissue engineering by using the inkjet printing technology, calcium peroxide microparticles, and alginate-hydrogel. The principal objective was to create scaffolds able to maintain cell viability by supplying oxygen when exposed to a hypoxic environment.

A modified off-the-shelf inkjet printer allowed printing calcium peroxide microparticles (< 40 μm) in a layer-by-layer fashion, controlling the amount and the pattern dispensed. Oxygen concentration generated by the hydrolytic decomposition of calcium peroxide was evaluated under hypoxic environments.

Three different concentrations of hydrophilic hydrogels composed of alginate and calcium chloride, were used to encapsulate the oxygen generating compounds, and delay the oxygen release. Hydrogels containing 16:1 alginate to CaO_2 allowed a continuous oxygen supply above 0.05 mol/m^3 for up to 120 hours. After 120 hours, degradation of the hydrogel occurred most likely because the continuous generation of hydrogen peroxide.

Fibroblast cells (L-cell) were seeded onto oxygen generating scaffolds, positive and negative controls scaffolds. Oxygen generating scaffolds and negative control scaffolds were incubated under hypoxic conditions, while positive scaffolds were cultured at standard conditions. Mitochondrial activity was evaluated by the MTS assay, where absorbance values were correlated directly to number of cells; they did not proliferate onto the scaffolds because of alginate hydrogel properties, but remained viable in the positive control with 68% after 120 hours. Viability was also assessed in the other groups after 120 hours of culturing. The viability of cells grown on the oxygen generating scaffolds was 60%, while cells frown on the negative control scaffolds were 21%. Live/dead assay corroborated these results; the highest cell density was observed on the surface of the oxygen-generating scaffolds in comparison with negative control scaffold.

The results suggest that these oxygen-generating scaffolds are able to create a continuous oxygen supply and improving cell viability under hypoxic incubation, nevertheless, are still far from recapitulating the normoxic environment needed for normal cell proliferation and differentiation. However, the scaffolds did improve cell viability, and this may be key for building large constructs of engineered tissues. One hypothesis would be that if scaffolds can maintain viability for an extended time, neovascularization will slowly restore normoxic conditions in implanted tissues. Vascularization into tissue scaffolds occurs approximately 42 μm per hour; therefore, alginate-hydrogel scaffolds that maintain cell viability for 120 hours would allow to implant a 5 mm thick of tissue, which would be an improvement of at least a factor of 5 over current technology.

Appendix 1

Calculation of area under the curve by numerical integration

The total oxygen generated was obtained calculating the area under the curve by numerical integration (trapezoidal rule) as described below:

$$\int_a^b f(x)dx \approx \frac{a-b}{2N}\sum_{k=1}^{N}[f(x_{k+1}) + f(x_k)]$$

Where:

(a – b) is the time spacing, and [$f(x_k)$, $f(x_{k+1})$] are the sensor measurements at time *a* and *b* respectively.

APPENDIX 1.1

Data used for calculation of oxygen generated by CaO_2/Medium proportion: 0.02%, 0.10% and 0.20% is showed below. The background oxygen level was removed to obtain only oxygen generated by calcium peroxide.

0.02% of CaO_2/medium		
Time (hours)	Oxygen measurements (mol/m^3)	Numerical Integration (mol/m^3)
0.2500	0.0822	0.0103
0.3500	0.0741	0.0181
0.4333	0.0831	0.0246
0.5000	0.0938	0.0305
0.6667	0.0816	0.0451
0.8333	0.0716	0.0579
1.0000	0.0731	0.0700
1.5000	0.0553	0.1021
2.0000	0.0563	0.1300
2.5000	0.0378	0.1535
3.0000	0.0447	0.1741
3.5000	0.0388	0.1950
4.0000	0.0263	0.2112
4.5000	0.0216	0.2232

0.1% of CaO_2/medium		
Time (Hours)	Oxygen measurement (mol/m^3)	Numerical Integration (mol/m^3)
0.2500	0.1888	0.0236
0.3417	0.1850	0.0407
0.4167	0.1769	0.0543
0.5000	0.1866	0.0694
0.6667	0.1553	0.0979
0.8333	0.1478	0.1232
1.0000	0.1447	0.1476
1.3333	0.1603	0.1984
2.0000	0.1194	0.2916
2.5000	0.1209	0.3517
3.0000	0.1288	0.4141
3.5000	0.1275	0.4782
4.0000	0.1172	0.5394
4.5000	0.1181	0.5982

0.2% of CaO_2/medium		
Time (Hours)	Oxygen measurements (mol/m^3)	Numerical Integration (mol/m^3)
0.2500	0.4306	0.0538
0.4167	0.3506	0.1189
0.5833	0.3041	0.1735
0.7500	0.2869	0.2227
1.0000	0.2431	0.2890
1.6667	0.2213	0.4438
2.0000	0.2003	0.5141
2.5000	0.2219	0.6196
3.0000	0.2238	0.7310
3.5000	0.1763	0.8310
4.0000	0.1703	0.9177
5.0000	0.1794	1.0925
6.0000	0.1606	1.2625
7.0000	0.1588	1.4222

5.0000	0.0191	0.2333
5.6667	0.0144	0.2445
6.1667	0.0119	0.2510
6.8333	0.0100	0.2583
7.3333	0.0097	0.2633
8.5000	0.0081	0.2736

5.0000	0.1059	0.6542
5.6667	0.1203	0.7296
6.1667	0.1125	0.7879
6.8333	0.0941	0.8567
7.3333	0.0947	0.9039
8.5000	0.0975	1.0160
14.0000	0.0588	1.4457
15.0000	0.0713	1.5107
16.0000	0.0822	1.5874
17.0000	0.0959	1.6765
18.0000	0.0528	1.7509
19.0000	0.0616	1.8081
20.0000	0.0622	1.8700
21.0000	0.0547	1.9284
22.0000	0.0281	1.9698
23.0000	0.0197	1.9937
24.0000	0.0113	2.0092
25.0000	0.0066	2.0181

14.0000	0.0953	2.3115
15.0000	0.1188	2.4185
16.0000	0.1853	2.5705
17.0000	0.2144	2.7704
18.0000	0.1925	2.9738
19.0000	0.1369	3.1385
20.0000	0.1088	3.2614
21.0000	0.0619	3.3467
22.0000	0.0500	3.4026
23.0000	0.0531	3.4542
24.0000	0.0466	3.5040
25.0000	0.0375	3.5461
26.0000	0.0281	3.5789
27.0000	0.0203	3.6031
28.0000	0.0131	3.6198
29.0000	0.0097	3.6312
30.0000	0.0084	3.6403

APPENDIX 1.2

Data used for calculation of oxygen generated by scaffolds with relative concentration of 16:1 alginate CaO_2 is showed below. The background oxygen level was removed to obtain only oxygen generated by calcium peroxide.

Scaffold relative concentration 16:1		
Time (Hours)	Oxygen measurement mol/m^3	Numerical Integration mol/m^3
0	0	0
1	0.0469	0.0234
23	0.0516	1.1063
46	0.0491	2.2635
72	0.0472	3.5149
96	0.0431	4.5987
120	0.0397	5.5925
144	0.0016	6.0875

Appendix 2

MTS assay from Promega

DESCRIPTION

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The MTS assay contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and an electron coupling reagent (phenazine ethosulfate; PES).

The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Assays are performed by adding a small amount of the MTS directly to culture wells, incubating for 1–4 hours and then recording the absorbance at 490nm. The quantity of formazan product as measured by absorbance at 490nm is directly proportional to the number of living cells in culture.

GENERAL PROTOCOL

1. Thaw the CellTiter 96® AQueous One Solution Reagent.
2. Pipet 20µl of CellTiter 96® AQueous One Solution Reagent per each 100µl of culture medium into each culture well.
3. Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO_2 atmosphere.
4. Record the absorbance at 490nm

Vita

Daniel Reyna Soriano was born in Ciudad Juarez, Chihuahua, Mexico, Daniel was the only son of Daniel Reyna Diaz and Maria Rosa Soriano Amaro. In the spring of 2006, Daniel earned his Bachelor of Science degree in Electrical and Mechanical Engineering in his Hometown University "Instituto Tecnologico de Ciudad Juarez" in 2006. He received his Master of Science degree in Mechanical Engineering in 2011 from The University of Texas at El Paso. In 2011 he joined the doctoral program in Biomedical Engineering at The University of Texas at El Paso.

Dr. Reyna Soriano receives a scholarship from Chihuahua State, Mexico, for his Masters and Doctoral studies at The University of Texas at El Paso.

During his Master's studies in the University of Texas at El Paso, he started to be interested in Tissue Engineering. While pursuing his doctoral degree, Dr. Reyna Soriano worked as a research assistant and assistant instructor. In his last semester he jointed to Biosense Webster as a full time staff member to develop new catheters for cardiovascular applications.

Dr. Reyna Soriano has presented his research at international conference meetings including Biomedical Society, NSF Research and Innovation, and Digital Fabrication. Additionally Dr. Reyna Soriano published his research in NIP & Digital Fabrication journal, and he participated in book chapters for Cambridge University Press, and Elsevier Inc.

Dr. Reyna Soriano's dissertation, Inkjet Bioprinting of Solid Peroxides for Constructing Oxygen Generating Scaffolds to Improve Cells Viability and Growth under Hypoxic Environment, was supervised by Dr. Thomas Boland.

Permanent address: Region de Bolonia 1301
Ciudad Juarez, Chihuahua, México, 32563

This dissertation was typed by Daniel Reyna Soriano