2011-01-01

Bioprinting Oxygen Releasing Microparticles for Improved Cell Tissue Viability

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

BIOPRINTING OXYGEN RELEASING MICROPARTICLES FOR IMPROVED CELL TISSUE VIABILITY

DANIEL REYNA SORIANO

Department of Mechanical Engineering

APPROVED:

_____
Tao Xu, Ph.D., Chair

_____
Connie Gomez, Ph.D.

_____
Bill Tseng, Ph.D.

_____
Patricia D. Witherspoon, Ph.D.
Dean of the Graduate School

To my lovely wife, and my parents

BIOPRINTING OXYGEN RELEASING MICROPARTICLES FOR IMPROVED CELL TISSUE VIABILITY

by

DANIEL REYNA SORIANO, BS

THESIS

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

MASTER OF SCIENCE

Department of Mechanical Engineering

THE UNIVERSITY OF TEXAS AT EL PASO

May 2011

ACKNOWLEDGMENTS

First and foremost, I owe my deepest appreciation to Dr. Tao Xu for his support, guidance, friendship, and encouragement throughout this project. I would also like to express my sincere gratitude to my committee members: Dr. Connie Gomez, and Dr. Bill Tseng.

Additionally, I would like to thank Jorge Rodriguez for assistance throughout this project. Moreover, the following individuals for their support and encouragement to gain new strengths for the completion of the project in different ways: Eduardo Rios, Ricardo Espinoza, and Sunny Ambure.

In addition, I would like to thank Dr. Thomas Boland, Dr. Louis Everett, 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 Mrs. Guadalupe Chacón Monarrez, Mrs. Silvia Muñiz, and Mrs. Flor Marquez. 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.

Bioprinting Oxygen Releasing Microparticles for Improved

Cell Tissue Viability

ABSTRACT

by

DANIEL REYNA SORIANO

A major challenge in regenerative medicine/tissue engineering is the difficulty of providing adequate oxygen to all cells within implanted engineered tissues before full vascularization is achieved. To overcome this limitation, a variety of oxygen-releasing particles have been developed to improve cell and tissue survival. However, most of these particles are used in random mixtures within scaffolding materials, which usually leads to an uneven distribution of oxygen in bioengineered tissues. An ideal oxygen supply requires a precise depositing control of oxygen-releasing particles in scaffolds. Cell bioprinting is a novel tissue fabrication approach, in which the bio-printer can be programmed to deposit cells and/or biomaterials of various types and sizes in a very precise manner. In this study we have applied the inkjet printing technology to deposit calcium peroxide (CaO_2) using various designed concentrations. On top of that, a hydrogel was deposit to provide encapsulation enabling progressive oxygen release. Various rates were utilized searching for optimal cell viability and growth in a period of 10 days under hypoxic environment (~1%O_2). This controlled oxygen-releasing platform (CaO_2 - two printing times scaffold) increase levels of cell viability by approximately 300% in comparison to control samples without CaO_2 present during the experiment. In

consequence, these biomaterials were able to extend cell viability and growth under hypoxic conditions. Results indicate the use of bio-printing technology to precisely deposit oxygen exerting biomaterials in vitro. Such technology shows promising application in regenerative medicine/tissue engineering where currently oxygen diffusion limits the engineering of tissue implants.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

1. INTRODUCTION

Tissue engineering has attracted wide interest as a potential method to alleviate the shortage of transplantable organs. To date, almost all of the successfully engineered tissues/organs have relatively thin and/or avascular structures (e.g., skin, cartilage, and bladder), where post-implantation vascularization from the host (angiogenesis) is sufficient to meet the implant's demand for oxygen and nutrients. Vascularization remains a critical obstacle impeding attempts to engineer thicker, metabolically demanding organs, such as heart and liver. For regeneration of damage tissues, porous scaffolds loaded with living cells and growth factors have been investigated. A big challenge within this area is the difficulty of providing sufficient oxygen to cells within implanted engineered tissues before full vascularization is achieved. At early implantation stage, oxygen supply is limited to adjacent parts of the scaffold, leaving the further parts of it with restricted or no oxygen at all jeopardizing the viability and proliferation of the seeded cells [1]. This has been a critical limiting factor for developing readily large functional tissues for clinical applications[2].

To overcome the lack of oxygen limitation, several strategies have been employed to improve the delivery of oxygen and improve the viability of the implanted tissue. To improve the neovascularization, angiogenic growth factors have been used, such as vascular endothelial growth factor, which is a potent endothelial cell-specific mitogen [3]. Even though the effectiveness of an improved angiogenic response has been demonstrated in tissue systems, the angiogenesis rate could not be accelerated, hence limiting the size of implants [4]. Another approach to maintain tissue viability in

vivo is to place the engineered tissue adjacent to a heavily vascularized tissue such as omentum in order to achieve adequate vascularization [5]. However, this approach may not always be feasible because the target implantation site may not be in close proximity to a heavily vascularized tissue. Other studies have examined the use of synthetic oxygen carriers such as perfluorocarbons and crosslinked hemoglobin [6, 7]. Atala et al. have previously reported the synthesis of oxygen generating biomaterials [8]. This technology is based on chemical decomposition of solid sodium percarbonate to produce oxygen over a period of hours. Upon contact with water, solid sodium percarbonate decomposes producing oxygen as well as sodium and bicarbonate ions as biocompatible byproducts [9]. Previous authors have shown that oxygen generating biomaterials are able to maintain cell viability under hypoxic conditions and that this material preserves tissue survivability and prevents necrosis during ischemia [8].

In this study we investigated the possibility of incorporating oxygen generating biomaterials by the inkjet bioprinting technology into tissue engineered constructs to provide a sustained oxygen release over an extended period of time. We envision this would create a favorable environment for maintaining cells in a hypoxic environment.

2. LITERATURE REVIEW

2.1. Vascularization Challenge

A major limitation of all of tissue engineering is the lack of methods to produce tissues with the ability to provide vascular supply. The human left ventricle is typically 1-1.5 cm thick, yet diffusion can only supply nutrients to a depth of approximately 100μm. When an engineered tissue construct is implanted, the seeded cells will have a limited capacity to take up nutrients, most critically oxygen, and to clear metabolic products, leading to intensive apoptosis and necrosis. Several strategies have been proposed to induce vascularization of engineered tissues: (1) optimization of scaffold material and design to favor cell infiltration, (2) the incorporation of growth factors into the scaffolds to induce angiogenesis, (3) preseeding of the implant with mature or precursor endothelial cells, and (4) scaffold vascularization with the aid of host coronary circulation in vivo [10].

2.2. Angiogenic Growth Factors

Formation of new blood vessel was usually classified into two well-known embryological phenomenons: vasculogenesis and angiogenesis. Vasculogenesis is described as a primary in situ differentiation of endothelial cells from mesodermal precursors, leading to the formation of primary capillary poleaxe. Angiogenesis was thought to be exclusively the result of vessels sprouding from pre-existing vessels. But recently, Asahara et al. have showed that circulating endothelial cell progenitors are also involved in the angiogenic process [11]. Concerning collateral vessel formation, a third concept, called anteriogenesis has been introduced by Scahapper [12]. It consists

of the remodeling of small capillaries into larger arterioles, with a muscular layer. Nevertheless, those three entities require:

- Proliferation and migration with a spatial organization of endothelial cells
- Dissolution and regeneration of the vascular extracellular matrix, considered as vascular stake
- Participation of mesenchymal perivascular cells (smooth muscle cells and pericytes)

During post-natal life, angiogenesis has been reported to be implicated in physiological situations, including tissue turnover, wound repair, ovulation and placenta growth, but also in pathological situations such as ischemia (cardiac and peripheral arterial diseases), rheumatoid arthritis and neoplastic tumors [13].

Throughout the 1990s, converging research has permitted isolation of major growth factors, responsible for those different steps of angiogenesis: Vascular Endothelial Growth Factor (VEGF), fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF), Transforming Growth Factor (TGF), Hepatic Growth Factor (HGF) and Tumor necrosis Factor (TNF). Since angiogenic growth factors are members of an exponential growing family, and that angiogenesis becomes a very complex process, there is a real need for a classification of growth factors. Moreover, growth factors have great diversity of cellular and molecular targets [14].

2.3. Oxygen Generating Biomaterials

As another approach for improving oxygen supplied to engineered tissues, numerous biomaterials exerting oxygen by means of degradation are noteworthy.

Biomaterials include the use of oxygen rich fluids such as perfluorocarbons and silicone oils [15, 16]. Other approaches to maintaining tissue viability attempted include the use of synthetic oxygen carriers, sodium percarbonate (SPO), calcium peroxide (CPO), and so on.

2.3.1. Synthetic Oxygen Carriers

Bio-artificial oxygen carriers include modified human or animal hemoglobin-based carriers, stabilized hemoglobin tetramers, polymerized hemoglobin, conjugated hemoglobin, and liposome encapsulated hemoglobin. Other carriers also include recombinant hemoglobin or from transgenic studies. Synthetic oxygen carriers include lipid–heme vesicles, hemoglobin aquasoms, and perflurocarbon based carriers [17].

2.3.2. Calcium Peroxide

Calcium peroxide was used to generate oxygen within the scaffold to maintain viability over a period of several days. This compound has been used in the agriculture and aquaculture industries for many years as an oxygen enriching supplement to improve bacterial viability in soil by releasing oxygen over an extended period of time [18, 19]. When it comes in contact with water, it decomposes according to the following chemical equations [20].

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

[Equation 1]

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

[Equation 2]

In constructing the scaffold, one concern is that, since hydrogen peroxide is generated as an intermediate product in the calcium peroxide decomposition, residual reactive oxygen species may be present. So, catalase was added to the media to ensure the decomposition of any hydrogen peroxide byproducts generated [22]. Catalase was added directly to the media for convenience; however, for in vivo applications, one could envision incorporating catalase or other anti-oxidants directly into the biomaterial. Studies are currently ongoing to quantify how much residual hydrogen peroxide is generated by the scaffold [8].

2.4. Sodium Percarbonate

Another approach to prolonging cell survival until host neovascularization is achieved the synthesis of oxygen generating biomaterials [8 -Harrison, atala]. This technology is based on chemical decomposition of solid sodium percarbonate to produce oxygen over a period of hours. Upon contact with water, solid sodium percarbonate decomposes producing oxygen as well as sodium and bicarbonate ions as biocompatible byproducts [8]. Studies have been developed to incorporate such oxygen generating biomaterials into films of Poly(D,L-lactide-co-glycolide) (PLGA) to provide oxygen to cells for a period of 24 h[21].

2.5. Alginate

“Alginate” is a collective term for a family of polysaccharides produced by brown algae [22] and bacteria [23, 24] . Alginic acid was first discovered, extracted, and patented by Stanford [25]. This polysaccharide was recognized as a structural component of marine brown algae (Phaeophyceae), where it constitutes up to 40% of

the dry matter and occurs mainly in the intercellular mucilage and algal cell wall as an insoluble mixture of calcium, magnesium, potassium, and sodium salts [26]. The presence of alginate provides the mechanical strength and flexibility of the seaweed and, additionally, acts as water reservoir preventing dehydration once part of the seaweed has been exposed to air. Alginate can thus be regarded as having the same morphophysiological properties in brown algae as those of cellulose and pectins in terrestrial plants. The main industrial applications of alginate as a natural polymeric material are linked to its stabilizing, viscosifying, and gelling properties and its ability to retain water.[27]

Alginates meet all the requirements for their use in pharmaceutical and medical applications. They have been largely used in wound dressings, dental impression, and formulations for preventing gastric reflux. However, the most advanced biotechnological and biomedical application of alginate resides in its use as a hydrogel for cell immobilization for applications ranging from production of ethanol from yeast cells and of antibiotics or steroids [28] to transplantation and cell therapy [29-34]. In the latter case, alginate gel is used as a selective immune barrier to protect the transplanted cells from the host immune system [35].

Alginate-encapsulated islets have been studied for many years, and an ongoing clinical trial (Novocell) is using interfacially polymerized PEG-encapsulated islets (Fig. 1). The success of these trials is not yet known, but porcine islets have already been shown to be protectable in a short-term discordant xenotransplantation model. Interestingly, our own work has shown that even a molecular-scale PEG cage can

immunoisolate islets, and this has now been shown to eliminate insulin dependence in diabetic animals [35].

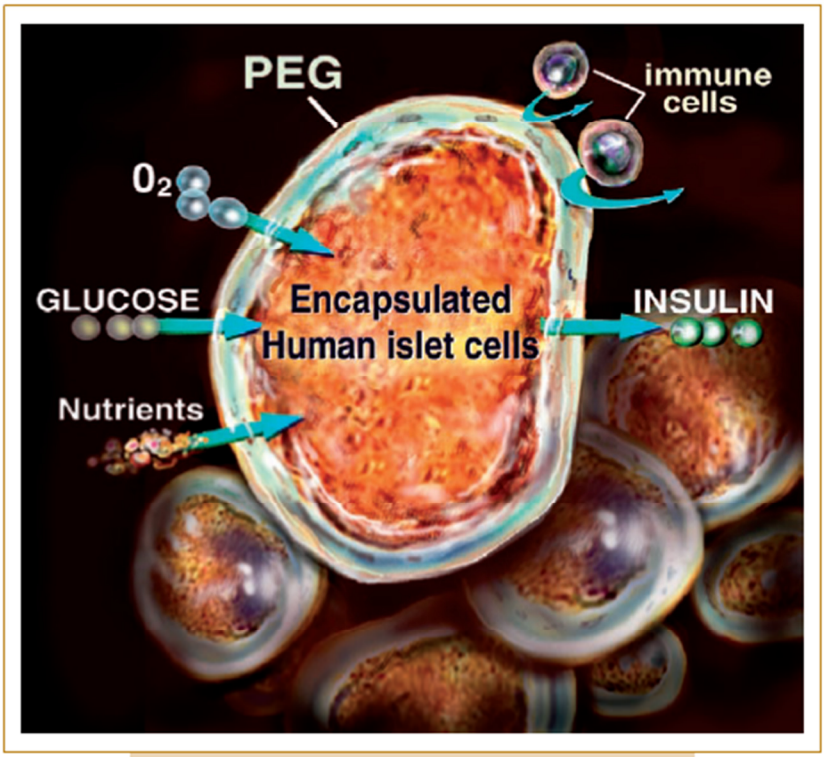

Figure 1. Interfacially polymerized PEG-encapsulated islets [35]

2.6. C2C12 Myoblast

Myoblast cells from Mus musculus (mouse) (C2C12 cell line) were utilized in the experiment to evaluate its behavior under progressive oxygen release. A myoblast is a type of embryonic progenitor cell that gives rise to muscle cells (myocytes) .The muscle cells can be skeletal muscle, smooth muscle, or cardiac muscle. The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins, with the help of a specified protocol [36].

2.7. Inkjet Bioprinting

Recently, the inkjet printing technique has attracted much attention as a useful tool for the fabrication of cellular patterns on a substrate. In this technique, precise target positions on a substrate can be assigned by computer-assisted deposition. Inexpensive commercially available printers can be used in these experiments with little modification. Several studies have shown the successful creation of cellular patterns on substrate by using this inkjet printing technique [37-43]. There are two main strategies, printing with living cells and printing with cell adhesion molecules [44].

Boland and coworkers reported that Chinese hamster ovary cells and primary embryonic hippocampal or cortical cells can be directly printed onto a substrate with a desired pattern without loss of cell function [37, 38]. The printing of cell adhesion molecules such as collagen or cell growth/differentiation factors such as FGF-2 and CNTF with a desired pattern onto a substrate has also been demonstrated using inkjet printers [40-42].

2.8. Microdrop Technologies [45]

Microdrop Technologies is the leading provider of equipment, software and services for advanced micro-dispensing and inkjet printing applications. The technology from microdrop is a versatile tool for liquid handling and material deposition (adhesives, coatings, waxes, nanoparticles, polymers, DNA, proteins, cells, etc.). The main 3 technologies (showed in the table 1) from Microdrop included:

1. Microdrop – Inkjet technology
2. Nanojet – Valve technology
3. Dropjet – Continous drop generation

Table 1. Microdrop Technologies

Characteristics	Microdrop – Inkjet Technolgy	Nanojet - Material Deposition by Valve Technology	Dropjet - Continuous Drop Generation
Single drop volumes*:	25 - 500 pl	> 200 nl	-
Droplet diameter*:	30 - 100 µm	-	60 – 200 µm
Volume variation*:	approx. 1 %	approx. 5 %	approx. 2 %
Viscosity range:	1 - 100 mPas (unheated)100 – 10,000 mPas (heated)	0 – 50 mPas	1- 50 mPas
Drop rate*:	0 - 2000/s	-	20 – 80 kHz
Max. throughput:	1 µl/s	approx. 2 ml/s	-
***depending on the liquid**			

Microdrop – Inkjet technology

The microdrop technology uses the principle of the ink jet printing technology. The core of the microdrop dispensing head consists of a glass capillary which is surrounded by a tubular piezo actuator. At one end the capillary is formed to a nozzle (diameter 30 to 100 µm). Applying a voltage pulse, the piezo actuator contracts and creates a pressure wave which propagates through the glass into the liquid. In the nozzle region the pressure wave accelerates of the liquid. A small liquid column leaves the nozzle, breaks off and forms a droplet which flies freely through the air. Depending on the nozzle sizes (30 to 100 µm) volumes of 25 up to 500 pl (corresponding to drop diameters of 35 to 100 µm) are generated.

A limitation of the microdrop inkjet technology is the low throughput associated with the fact that only use a single nozzle for dispensing any given substance; in contrast, with the several set of nozzles utilized by the proposed inkjet which allows a high throughput.

Nanojet - Material Deposition by Valve Technology

The Nanojet system is the right choice for applications where volumes from 200 nl and larger are needed to be dispensed. The Nanojet technology forms a liquid jet by means of a time/pressure-controlled dispenser system. Liquid from a pressurized reservoir is pressed into the dispenser head which consists of a micro valve and a nozzle. By opening the valve, liquid flows through the nozzle and forms a well-defined jet. The integrated micro valve switches the jet on and off. Since the micro valve switches at a very fast rate, volumes down to 200 nl can be dispensed. The special nozzle geometry forms a laminar jet, which is stable over a wide range before breaking off into droplets.

Dropjet - Continuous Drop Generation

Dropjet – a combination of Microdrop and Nanojet technology – allows the generation of a large number of drops with a well-defined size. This is of interest in applications like micro-encapsulation, aerosol research, spray and micro-sphere production.

A jet is generated by pressurized liquid flowing through a nozzle. The jet is modulated by an integrated piezo actuator causing defined fluctuations of the jet diameter at the nozzle outlet. The fluctuations grow over time, constrictions are built and after a certain distance the jet disintegrates into droplets.

The drop volume is defined by the flow rate, the frequency of modulation and the nozzle diameter of the system. The smallest possible drop size - approx. two times the size of the nozzle diameter - is defined by the smallest wave length of the periodic fluctuation. The maximum volume is limited by the stable separation of the drops. At larger distances of the constrictions the liquid tends to separate into more than one drop with different volumes.

3. HYPOTHESIS AND OBJECTIVES

3.1. Hypothesis

It is hypothesized that modified commercial inkjet printers HP697C can directly deposit oxygen micromaterials to create scaffolds for improved cell viability and proliferation.

3.2. Objectives

a) To demonstrate that oxygen micromaterials can be directly deposited by inkjet printing technology.

b) To study the effect of the printed oxygen microparticles on cellular viability and proliferation

4. MATERIALS AND METHODS

4.1. Calcium Peroxide Suspension

Calcium peroxide (CPO) powder (Aldrich, MO, USA) was deposited into a 50mL tube and mixed with 10mL of the bio-ink ethanol 200 proof (Aldrich-Sigma, MO, USA) to obtain a suspension relation of v/m with 1%wt of CPO. Then the CPO suspension was mixed with the vortex mixer (Fisher Scientific, PA, USA) and filtered with a 40µm filter (Fisher Scientific, PA, USA) prior to being dispensed in the printing cartridge to reduce clogging conditions during printing process.

4.2. Alginic Acid Suspension

Alginic Acid (AA) powder (Acros Organics, NJ, USA) was deposited into 50mL tube and mixed with 10mL of the bio-ink ethanol 200 proof (Sigma-Aldrich, MO, USA) in order to obtain a suspension relation of v/m with 1%wt of AA. Then the AA solution was filtered with a 40µm filter to reduce clogging conditions during printing.

4.3. Calcium Chloride solution

Calcium Chloride ($CaCl_2$) powder (Acros Organics, NJ, USA) was deposited into 50mL tube and mixed with distilled-sterilized water in order to have a relation of v/m with 1.4 %wt of $CaCl_2$. Then the $CaCl_2$ solution was filtered with a 40µm filter to clean the water.

4.4. Water preparation

Distilled water was sterilized by the autoclave machine, and filtered with a 40µm filter (Fisher Scientific, PA, USA) before usage as printing suspension.

4.5. Design Software

The printer patterns for the oxygen microparticles were designed using PowerPoint software (Microsoft Inc., WA, USA).

4.6. Printer and Cartridge Modification

The modifications to the HP printer model 697C (Hewlett-Packard, Palo Alto, CA) were limited to removing the rubber cleaners, which are used to clean the cartridges nozzles, were removed along with their respective springs use to maintain contact with the cartridge when needed; moreover, bypass the feed page sensor, and add a push button switch.

A black ink-jet cartridges (HP 29) were emptied of its contents, thoroughly washed, rinsed with a 70% ethanol solution, and distilled water and dried in a sterilized Labculture® Class II, Type A2 Biological Safety Cabinet (ESCO, PA, USA) before being filled with 100µL of microparticles suspension. This procedure proved to be an effective method for the cleaning and sterilization of the cartridges. This was determined by printing 4mm black dot pattern different times printing, and microparticles density difference were observed.

4.7. Volume Determination

In determining the amount of volume being dispensed by the inkjet printer an evaluation test was design to allow calculating the delivered volume by knowing the concentration of the solution and the mass being dispensed. Sodium chloride (Acros Organics, Geel, Belgium) was dissolve in distilled water (Millipore, MA, USA) in the

closest ratio to full saturation, 1:3. The solution was inkjet printed with a black cartridge and results were obtained.

The procedure for determining the volume was as follow.

1) Mass of the glass slides utilized to print the given solution was measured with laboratory balance model ALF 204 (Fisher Scientific, PA, USA).
2) Different patterns (see Figure 2.) were printed utilizing both solutions onto glass slides to correlate the dot size with the volume dispensed
3) Glass slides were exposed to 100 ± 15 ℃ at isotemp oven (Fisher Scientific, PA, USA) for at least 10 minutes to allow all the water to evaporate.
4) Mass of the glass slides with the remaining salt was measured with laboratory balance model (Fisher Scientific, PA, USA).
5) Knowing the solution concentration and dot size being print volume was determined.

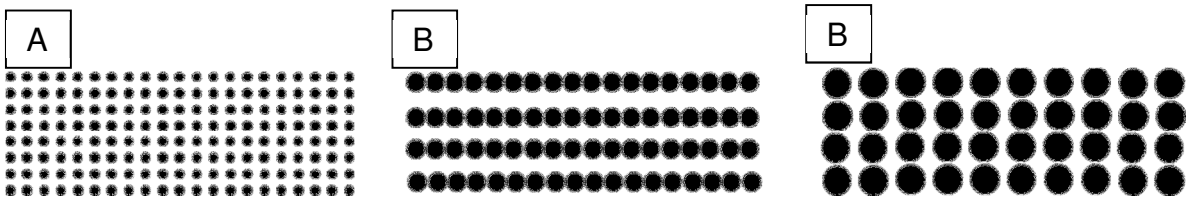

Figure 2. Patterns printed for volume determination. . (A) Dot size 0.05", (B) Dot size 0.10", (C) Dot size 0.15"

As control, a controlled volume was dispensed over a glass slide under a conventional micropipette deposition method (100 µl). Percentile error was obtained from this test to quantify the tolerance error of the printed parameters under both substances utilized.

4.8. Scaffold Construction

A 96 well-plate (BD Falcon, NJ, USA) was used as a substrate for the scaffold. The dot size for build the scaffold was of 4mm of diameter; five versions of scaffolds were printer with different concentrations of CPO. The scaffolds were constructed as follow:

a) CPO solution was printed into the well, creating 4 different versions, 2, 8, 16, 32 printing times.
b) AA was printed 32 times onto the same CPO pattern printed, and onto a new well in order to have a control sample with only hydrogel
c) Sterilized and filtered water were printed 32 times onto the previous patterns, providing humidity to the alginic acid prior to the reaction with the calcium chloride
d) Calcium Chloride was printed 32 times onto the previous patterns, in order to form the hydrogel and encapsulate the CPO.
e) Group samples were identified as shown in the Table 2

Table 2. Scaffold identification

Sample ID	CPO Printing Passes	Hydrogel Printing Passes (AA/CaCl2)
SC	No CPO	32/32
S1-2X	2	32/32
S2-8X	8	32/32
S3-16X	16	32/32
S4-32X	32	32/32

Printing passes were determined based on amount of oxygen required by C2C12 cells for optimal proliferation.

4.9. Cell Culture procedure

The cells culture protocol was as follow.

a) C2C12 mouse myoblast cell line where thawed and transfer into a 15mL tube (Fisher Scientific, Pittsburgh, PA) and added 10mL of high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% FBS (Gibco), 500 U/mL penicillin (Gibco), 500 mg/mL streptomycin (Gibco)
b) C2C12 cells were centrifuged (Eppendorf , Hamburg, Germany) for 5 min at 1000rpm
c) The supernatant was aspired with a vacuum pump (Thermo Fisher Scientific, Waltham, MA)
d) 5 mL of culture medium was added to the centrifuged cells
e) Cells were transferred to a 150mm tissue culture dish (Falcon, Franklin Lakes, NJ) and added 15 mL of medium
f) The cells were incubated (5%CO_2, 37^{o}C) for 3 days

4.10. Proliferation Assay

Cell proliferation assays is essential experiment in many tissue culture projects. To help those who need to determine cell viability, Promega has released the CellTiter 96® Aqueous One Solution Cell Proliferation Assay.

The CellTiter 96® Aqueous One Solution Cell Proliferation Assay is based on similar principles to the widely used MTT assay. The active component is a novel tetrazolium compound called MTS (similar to MTT and its derivatives), which is reduced to a colored formazan product. The amount of formazan product is directly proportional

to the number of living cells; therefore, cell proliferation or death can be quantified by reading the plate at 490 nm.

The cells were prepared to test in a 96-well plate. 20 µl CellTiter 96® Aqueous One Solution Reagent were added to 100 µl media in each well. Plates were incubated for 2-3 hours at 37°C, and results were obtained by reading at 490 nm in an absorbance plate reader. As various conditions (media, serum, pH, light exposure, presence of other chemicals, light exposure) can affect the background absorbance, the manufacturer recommends including 'no cell' controls to allow for background adjustment. Following this recommendation control sample was defined as hydrogel scaffold containing only medium.

After incubated the scaffolds, 80µL of MTS-Medium solution was transferred to a 96-well plate to read the absorbance. The absorbance was measured by the BIOTEK ELISA reader. Three lectures per scaffold were obtained.

4.11. Cell density selection

The cell density per well plate was defined by a test designed to identify the appropriate cell density to exceed the background value stated by the absorbance reader using MTS assay. Such test evaluates the absorbance value, using the MTS cell proliferations assay after incubating samples at hypoxia conditions for 24hrs. Three different cells densities were seeded onto hydrogels, having as a control sample a no cell seeded hydrogel. The cell densities chosen for the evaluation were 2500, 5000, and 10000 cells per well. The cell density with an absorbance value above the MTS background stated as 0.20 was chosen to perform the test of scaffold evaluation.

4.12. Cells counting

After culturing the C2C12 cells for 3 days, a hematocytomer was used to obtain the cell number per mL from the cultured 150mm petri dish. Cells density needed to prepare the final suspension of 100000 cell/mL.

The procedure to count the cells per mL was as follow.

a) 250 µL of the 5mL cell suspension, and 250 µL of trypan blue were transferred into a 1.8 mL tube
b) After mix and obtain a homogenous solution, 20 µL of the solution was transferred into each chamber of the hematocytometer
c) Cells density per mL was obtained counting them with the hematocytometer and microscope

4.13. Cell solution preparation

After culture the C2C12 cells, and obtaining the cell density by the Hematocytometer, the final solution was prepared as follow:

a) An aliquots cells suspension was transferred into a 50 mL tube containing culture medium in order to have a cell density of 100 000 cells/mL
b) 100 U/mL of Catalase (Bovine Fetal Liver, SIGMA, USA) was added to the cells solution.

4.14. Scaffold evaluation

To evaluate whether scaffolds containing CPO could enhance cell growth and viability, C2C12 mouse myoblast cell line were seeded onto SC (no CPO) and oxygenized scaffolds (S1-2X thru S4-32X) with a cell density of 10000 cells/scaffold, and the seeded scaffolds were placed in a hypoxic incubator (1% O2, 5% CO2) (BioSpherix, USA). All scaffolds were washed with PBS to improve cell seeding efficiency [8].

Then the scaffolds into the 96 well plate (Falcon,USA) by the bioprinting technology, 100 µL of cell suspension in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% FBS (Gibco), 500 U/mL penicillin (Gibco), 500 mg/mL streptomycin (Gibco) was seeded on each scaffold. In addition, some aliquots of cells contained 100 U/mL catalase (from bovine liver, Sigma, USA). Catalase was added to react with hydrogen peroxide byproducts of metabolism and degradation [8]. The cells were seeded in a hypoxic glovebox system (1% O2, 5% CO2) (BioSpherix, USA) using nitrogen (N2) purged cell suspension to have hypoxia conditions since the initial time. The number of viable cells in each scaffold in culture was estimated using an MTS assay, which measures mitochondrial activity of cells.

To compare cell growth in SC (no CPO) and oxygenized scaffolds (S1-2X, S2-8X, S3-16X, S4-32X), cells were seeded on each type of scaffold using the above procedure and catalase containing culture medium. These scaffolds were cultured up to 10 days in a hypoxic incubator. The culture medium was carefully changed every 3 days in a hypoxic glovebox system (1% O2, 5% CO2) (BioSpherix, USA) using nitrogen (N2)

purged culture medium to prevent reoxygenation of the medium. The viable cell number in the scaffolds after culturing for 1, 3, 5, 7 and 10 days was estimated by an MTS cell proliferation assay.

4.15. Statistics

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

5. RESULTS AND DISCUSSION

5.1. Volume Determination

To determine the volume, of such small fluid dispensing, a control test was used to dispense 100 µl of a sodium chloride (NaCl) solution onto glass slides. The practical volume obtained for the NaCl solution was 91.00 µl which represent a percentile error of 8.99.

Three different patterns undergoing different diameter size dots were printed onto a glass slides and volume was determine by prior method described. The patterns, illustrated in Figure 2, were of 0.05, 0.10, and 0.15 inches of diameter according to the software which ultimately corresponded to an actual printed area of 1.24, 5.30, and 11.87 mm^2, respectively. Volume resulted in 4.84, 27.76, and 60.16 nanoliters in the same order. Data was 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 polynomial trend line was found to be the best line fit (regression line) (R-squared = 99.89), which resulted in a polynomial equation that describes the behavior of the volume with respect to the printed area. The resulted equation was:

$$Volume\ (y) = 5.1307\ area(x) - 0.4467$$

[Equation 3]

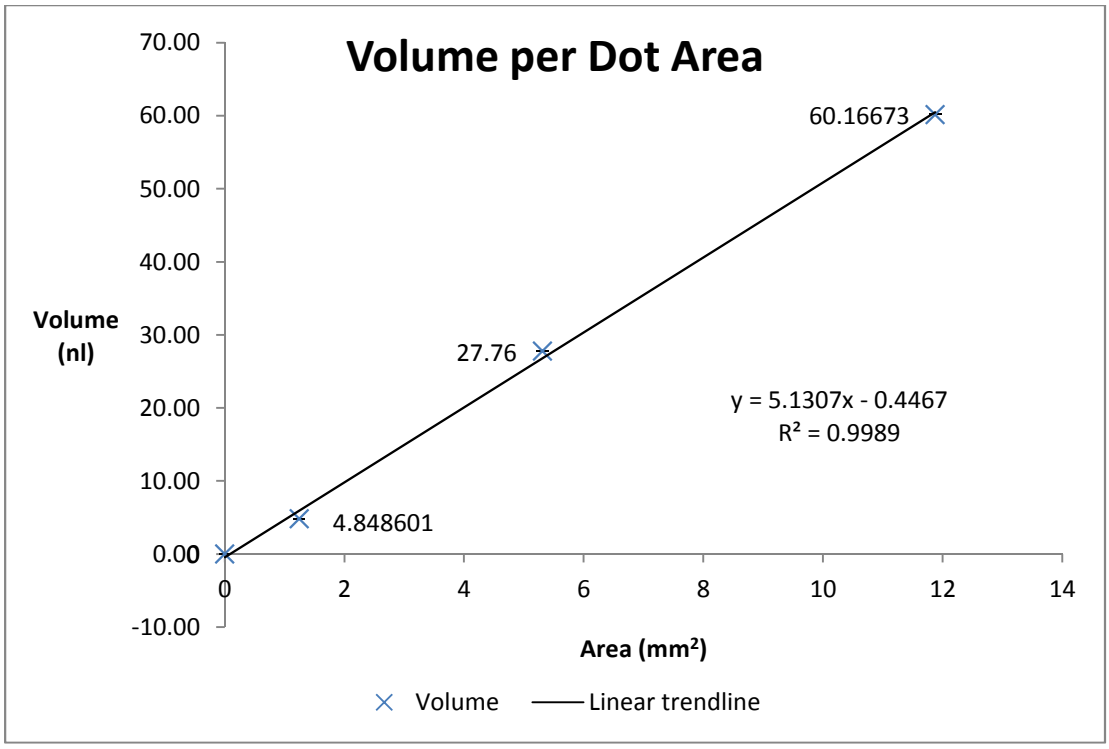

Figure 3 .Area vs. Volume per dots scatter diagram. Polynomial fit trend line. The equation 3 describing the trend line allows us to predict volume knowing the printed area.

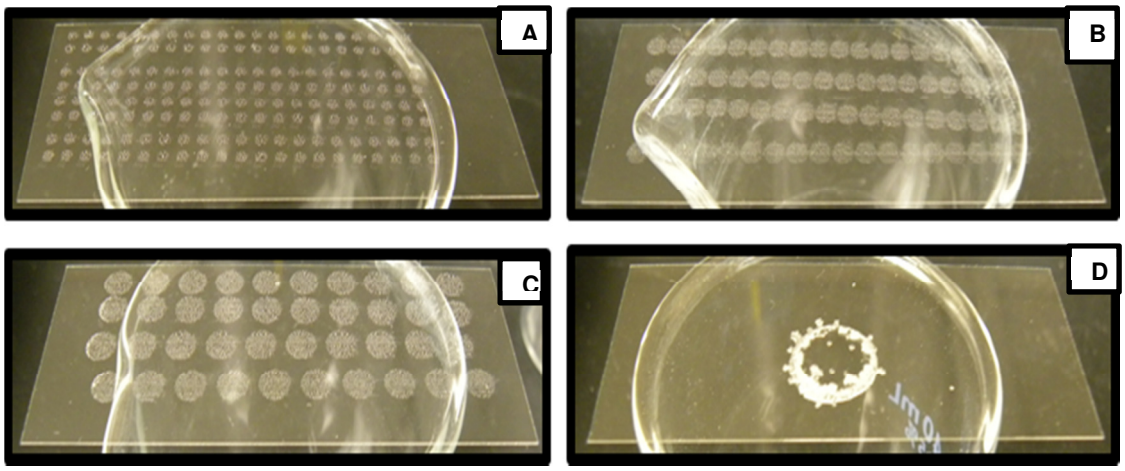

Figure 4. Patterns printed for volume determination. (A) Dot diameter 0.050". (B) Dot diameter 0.100". (C) Dot diameter 0.150". (D) Control, 100µL deposited manually

Table 3. Volume and mass relation of CPO printed

CPO		
Printing Times	Volume nL	Mass ng
2	128.05	8.83
8	512.22	35.34
16	1024.44	70.68
32	2048.88	141.47

Table 4. Printing times, Volume, and Mass relation, for hydrogel formation

Hydrogel			
Solution	Printing Times	Volume µL	Mass ng
Alginic Acid	32	2.05	88.1
Distilled Water	32	2.05	
Calcium Chloride	32	2.05	

5.2. Scaffold construction

Cylindrical SC (no CPO) and oxygenized scaffolds (diameter 4mm) were constructed by the bioprinting technology. Four different versions of scaffolds were constructed, S1-2X, S2-8X, S3-16X, and S4-32X (See table 2), and all of them were encapsulated by the hydrogel that formed by printing 32 times the following: AA, Sterilized-Filtered water, and CaCl2 (See table 2). One scaffold control was constructed containing only hydrogel.

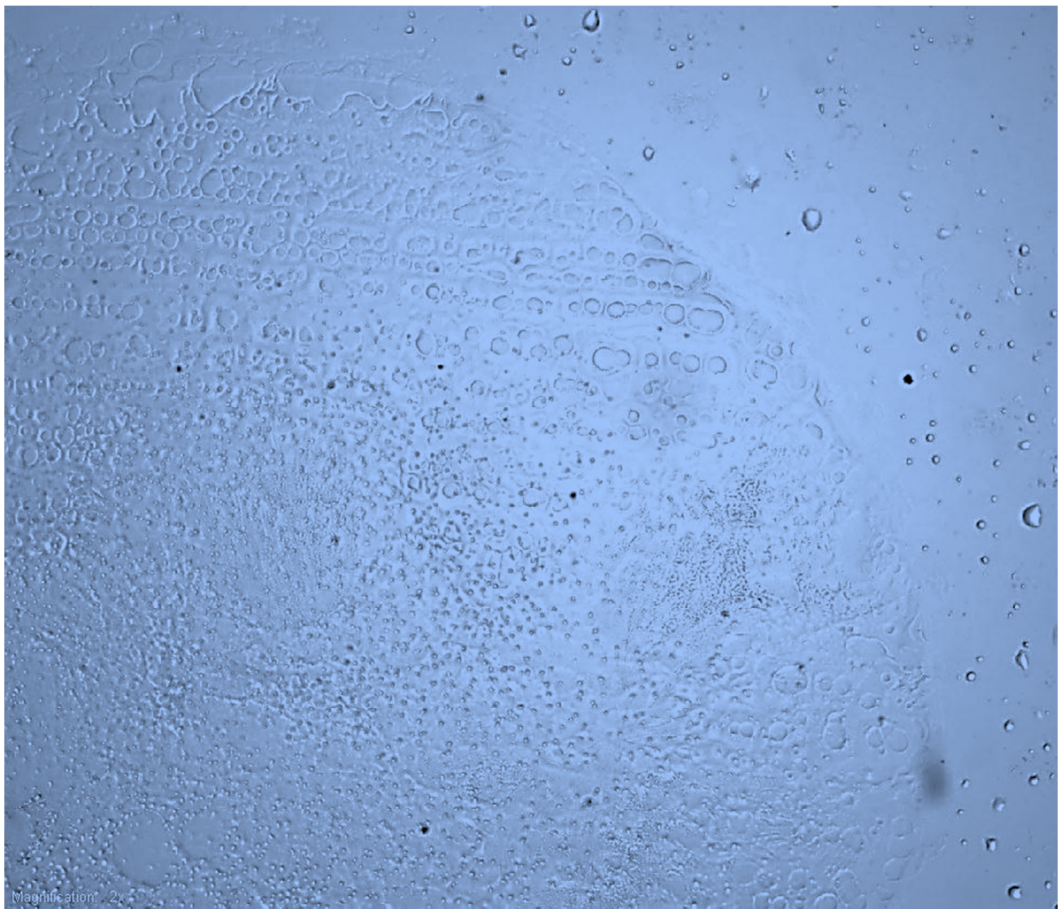

Figure 5. Hydrogel formation

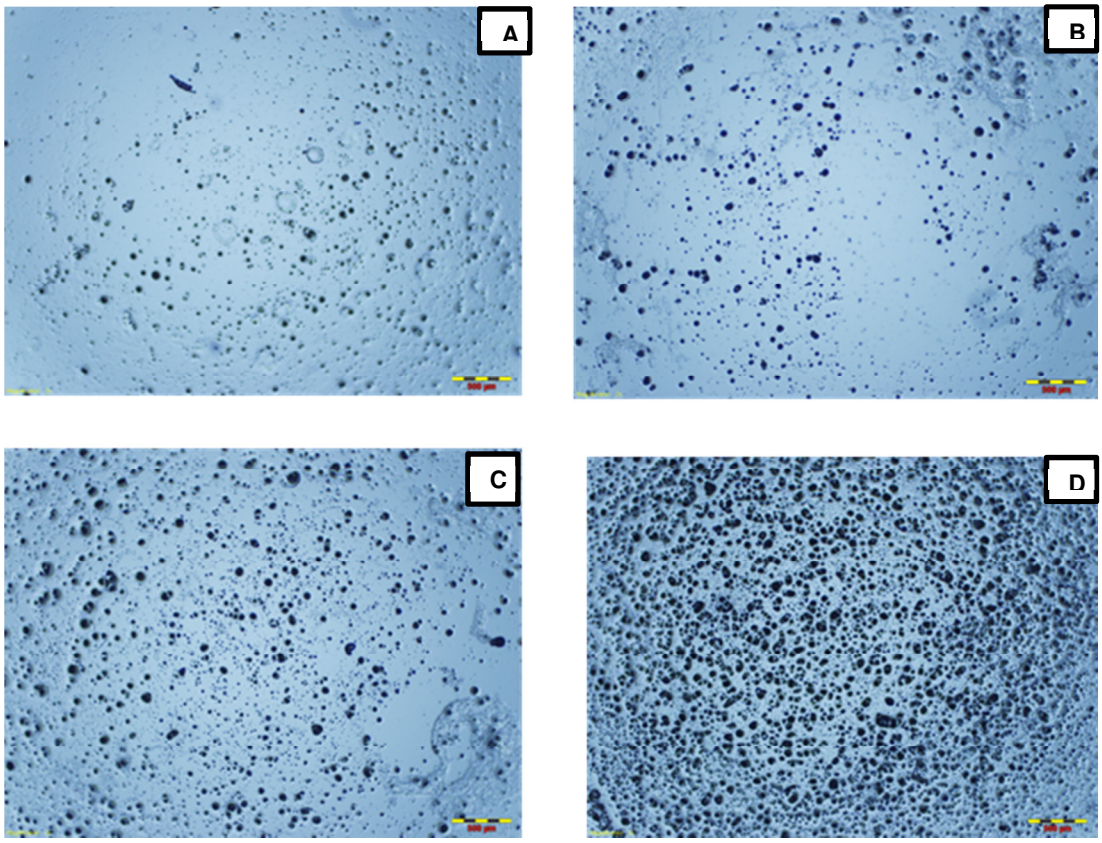

Figure 6. CPO encapsulated. (A) 2 times overprinted, (B) 8 times overprinted, (C) 16 times overprinted, (D) 32 times overprinted

.

5.3. Cell Density Selection

The cell density selection was obtained seeding onto hydrogel three different cell densities, 2500, 5000, 100000 cells per scaffold, and one as a control with no cells. Then the scaffolds were placed in a hypoxic incubator (1% O2, 5% CO_2) for 24 hrs. After incubate the cells for 24hrs, the medium was removed from the scaffolds, and washed with PBS. 100 µL of fresh medium and 20 µL of MTS solution were added to each scaffold, prior to incubate for 2 hrs. After incubation, 80 µL of medium-MTS solutions were transferred to a 96 well plate. Three lectures of absorbance per scaffold were obtained by the ELISA absorbance reader machine. A statistically significant difference ($P<0.05$) was observed in the samples with a cell density of 10000 cells/scaffold with respect the control sample (no cells), results shown in figure 7. The figure 8 illustrates the cells attached to the scaffold, and confirms the absorbance values obtained by the MTS assay.

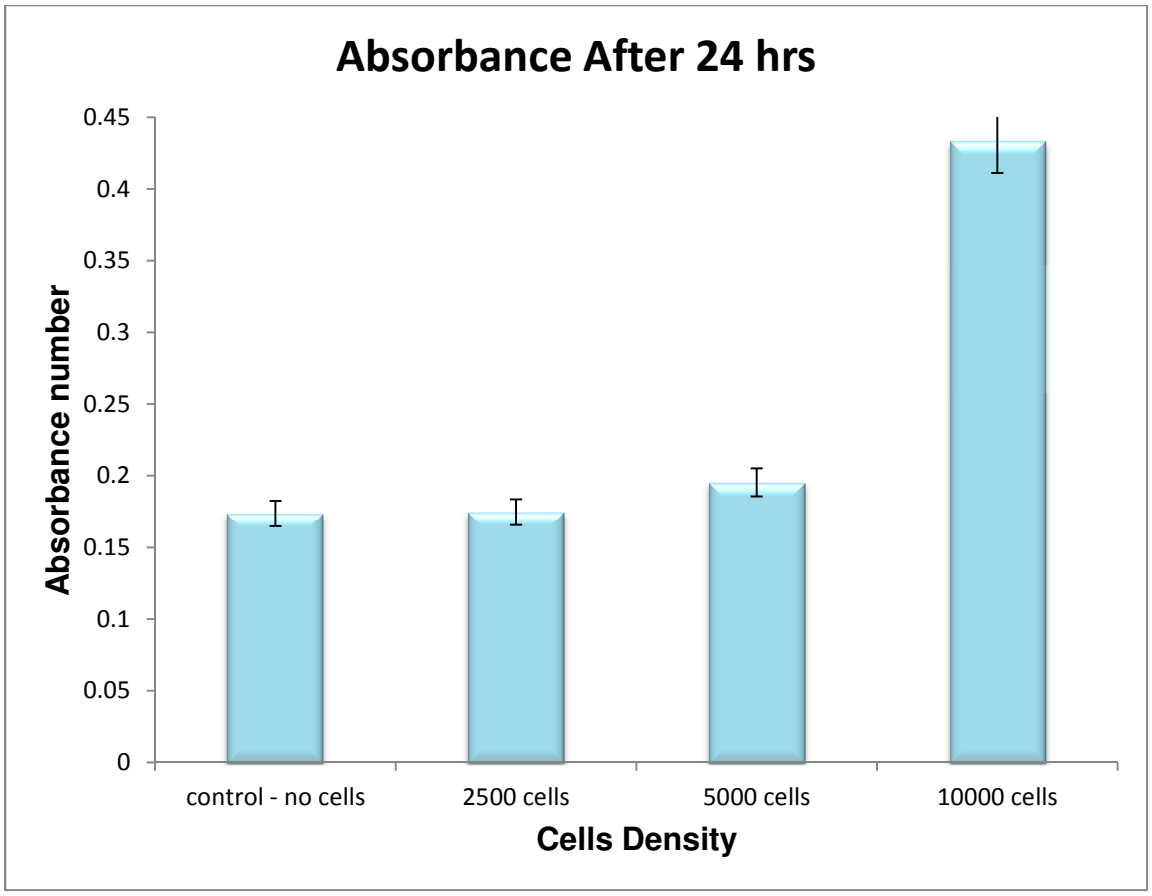

Figure 7. Absorbance values to determine cell density. The results shown that cell density of 10000cells/scaffold has an absorbance value statistical different with respect the control (P<0.05).

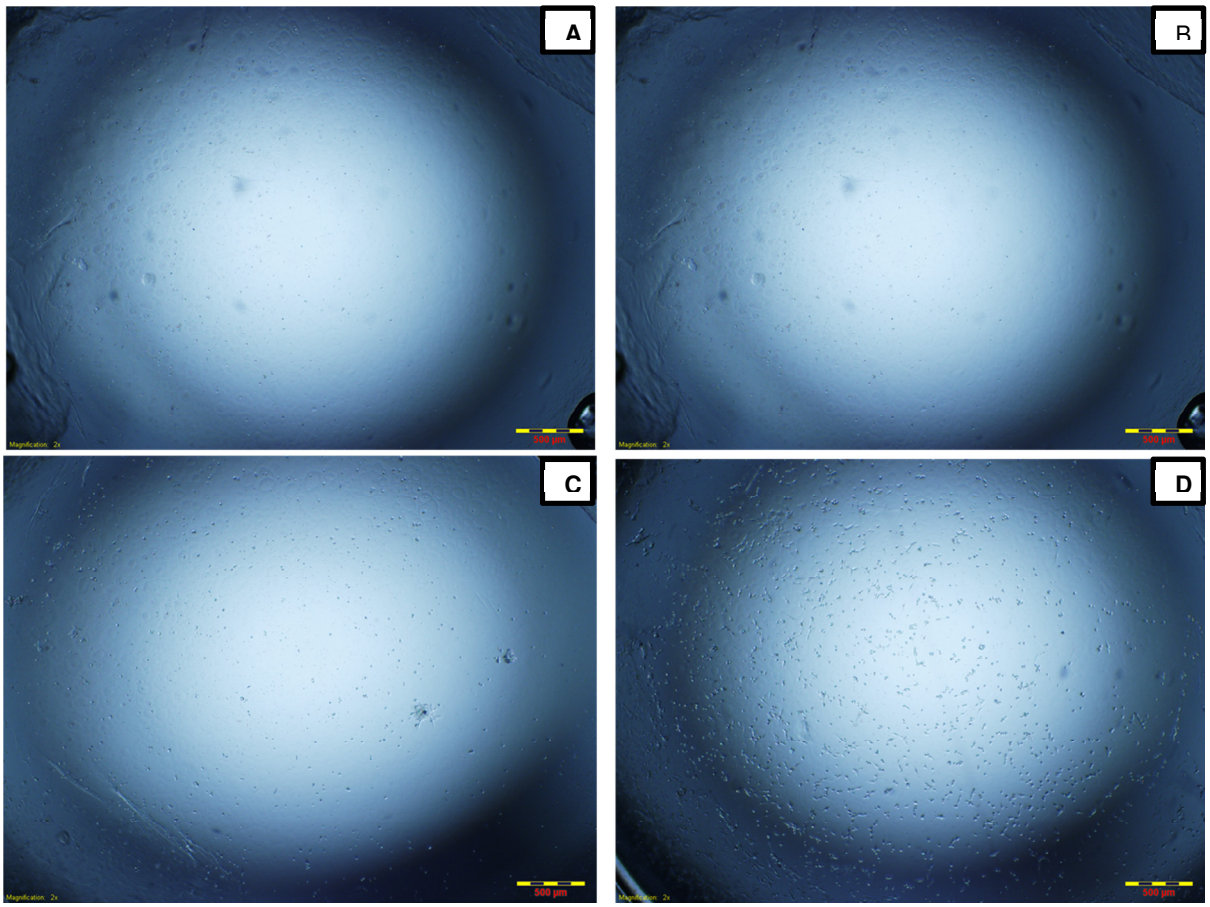

Figure 8. Cell density selection. Cells onto scaffold washed with PBS after incubate for 24hrs under hypoxic conditions. The cell density evaluated was, (A) Control – no cells, (B) 2500cells, (C) 5000 cells, and (D) 10000 cells.

5.4. Cell Culture and counting

Cell viability and number were determined by hemocytometry using Trypan blue exclusion. C2C12 mouse muscle cells were cultured for three days using a cell culture 150mm petri dish, the cells confluence is showed in figure 8. The cell density was obtained using the hematocytomer method, as shown in figures 9 and 10. The cell density of $1.35x10^6$cells/mL was obtained from 3 days of culture.

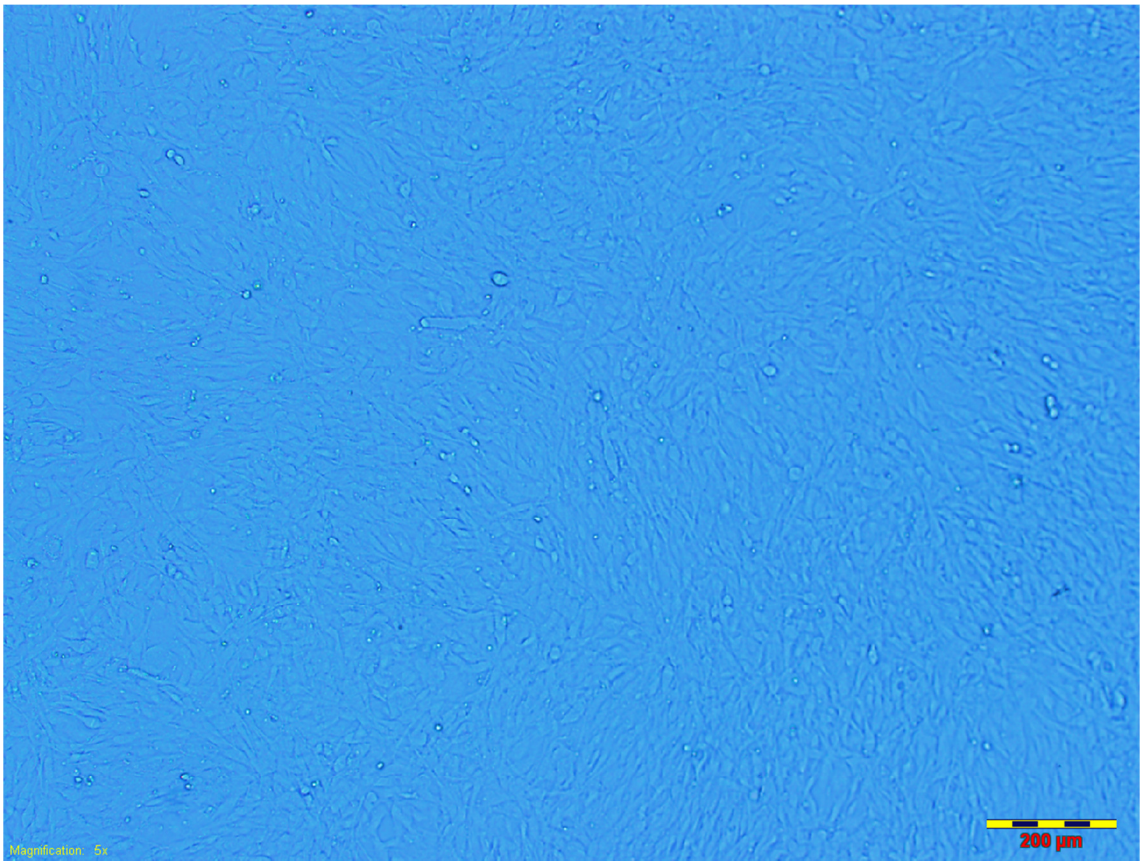

Figure 9. C2C12 cells confluent after 3 days of culture.

To prepare the hemacytometer, the mirror-like polished surface and the coverslip was carefully cleaned with lens paper and ethanol. The coverslip was placed over the

counting surface prior to adding the cell suspension. The cell suspension was introduced into one of the V-shaped wells using a pasteur pipet, allowing the area under the coverslip to fill out by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered (20µL). The counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power. One entire grid on standard hemacytometers with Neubauer rulings was captured as shown in figure 9 at 40x (4x objective).The main divisions separate the grid into 9 large squares. Each square has a surface area of $1mm^2$, and the depth of the chamber is 0.1mm. Each square of the hemacytometer (with cover slip in place) represents a total volume of 0.1 mm^3or 10^{-4} cm^3. Since 1 cm^3 =1 ml, the subsequent cell concentration per ml can be determined per excel calculation illustrated in figure 10.

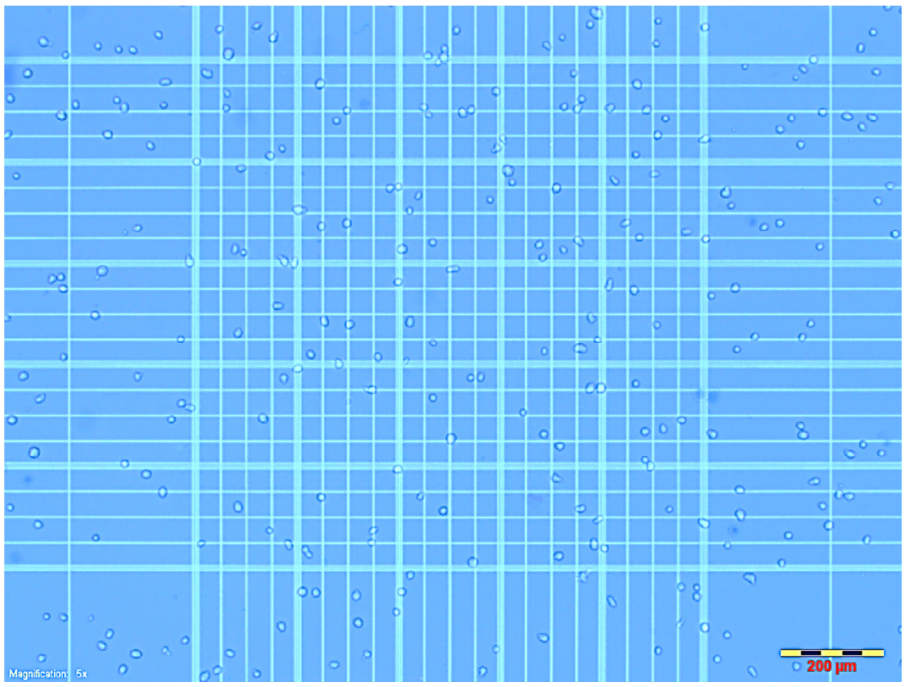

Figure 10. Hematocytometer containing C2C12 Cells

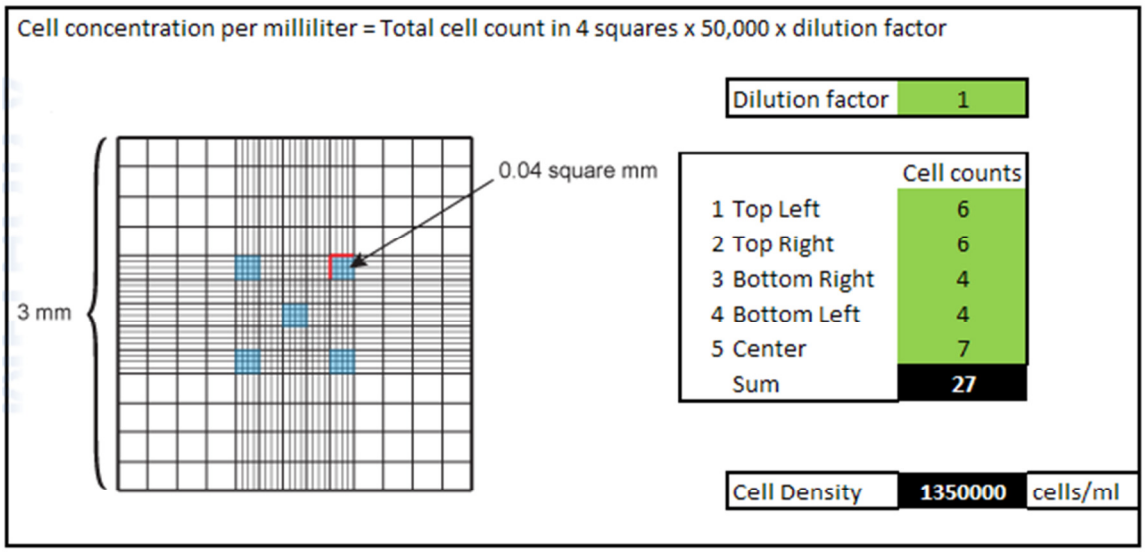

Figure 11. C2C12 cell counting method

5.5. Cell Solution preparation

To obtain a cell suspension of 100000cells/mL, 74µL of the cell suspension ($1.35x10^6$cells/mL) was transferred into a 50mL, containing 29.926mL of culture medium, obtaining 30mL of cell suspension with a cell density of 100000cells/mL. 100U/mL of catalase (Fetal Bovine Liver) was added to inhibit the hydrogen peroxide.

5.6. Scaffold Evaluation at hypoxic incubation

The C2C12 mouse muscle cells were seeded onto the SC (control) and oxygenized scaffolds (Samples S1-2X through S4-32X) scaffolds with a cell density of 10000cells/scaffold. To evaluate the effect of CPO, cells seeded onto control and oxygenized scaffolds were cultured under hypoxia conditions (1%O2, 5%CO_2) for 10 days and the metabolic rates of cells within each scaffold were estimated using MTS assay, as shown in figure 11.

The results at day 1 confirmed that the test was initiated under the same conditions for all the scaffolds, and the absorbance value was above the background (0.2). The values obtained through the days 3 and 5 were not statistically different ($P>0.05$), because the hydrogel thickness did not allow the oxygen diffusion. The cells on the different scaffolds proliferated similarly until day five ($P>0.05$), due to the cellular oxygen reserve obtained prior to their placement in a hypoxic incubator. Oxygen diffusion started at day seven, enhancing the cell proliferation on scaffold S1-2X. On scaffold S4-32X the oxygen rate was toxic for cells, while in the control sample the cell viability did not change due to the oxygen reserved. At day 10 the absorbance values dramatically decreased because the oxygen reserve was finished. The absorbance value in the control sample (SC) decreased below the background (no live cells), while the cell viability in the scaffold S1-2X had an absorbance value above to 0.6. The absorbance values on the scaffolds S2-8X, S3-16X, and S4-X32 were not statistically different ($P>0.05$), and were between the value of scaffold SC and the scaffold S1-2X.

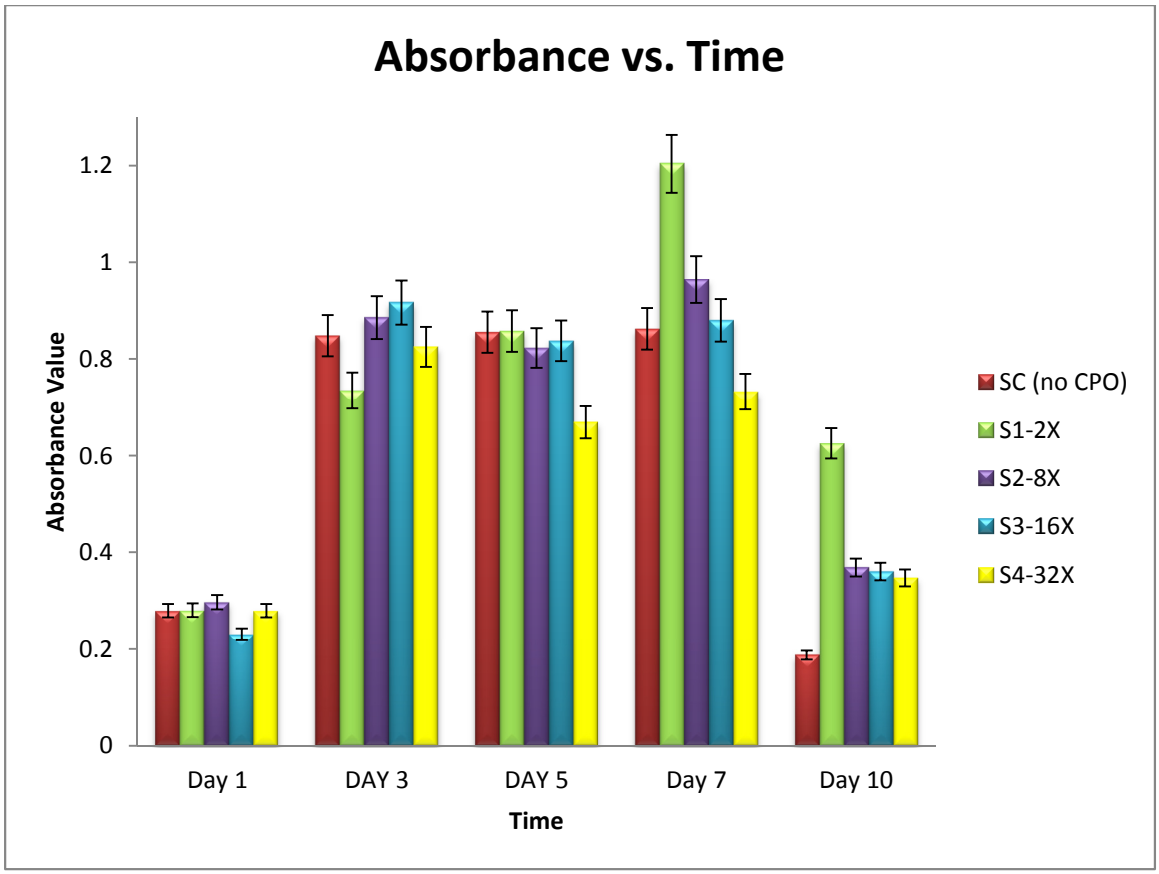

Figure 12. Absorbance value over time at days: 1, 3, 5, 7, 10. After day 7 the S1-2X scaffold showed higher cell viability and proliferation than S2-8X, S3-16X, and S4-32X.

The figure 13 illustrate that the scaffolds had a similar efficiency prior the day 7 due to the hydrogel thickness did not allow the oxygen diffusion. At the day 7 the scaffold S1-2X had approximately 50% more viability than scaffold S2-8X, S3-16X, S4-32X, and SC (no CPO). By the day 10 the scaffold S1-2X had an absorbance number approximately 330% higher than the SC scaffold, and approximately 130% with respect the scaffolds S2-8X, S3-16X, S4-32X. The results shown that the oxygen rate from the scaffolds S1-2X was more adequate than the oxygen rate from the scaffolds S2-8X thru S4-32X. The figure 14 confirms the absorbance values at day 10.

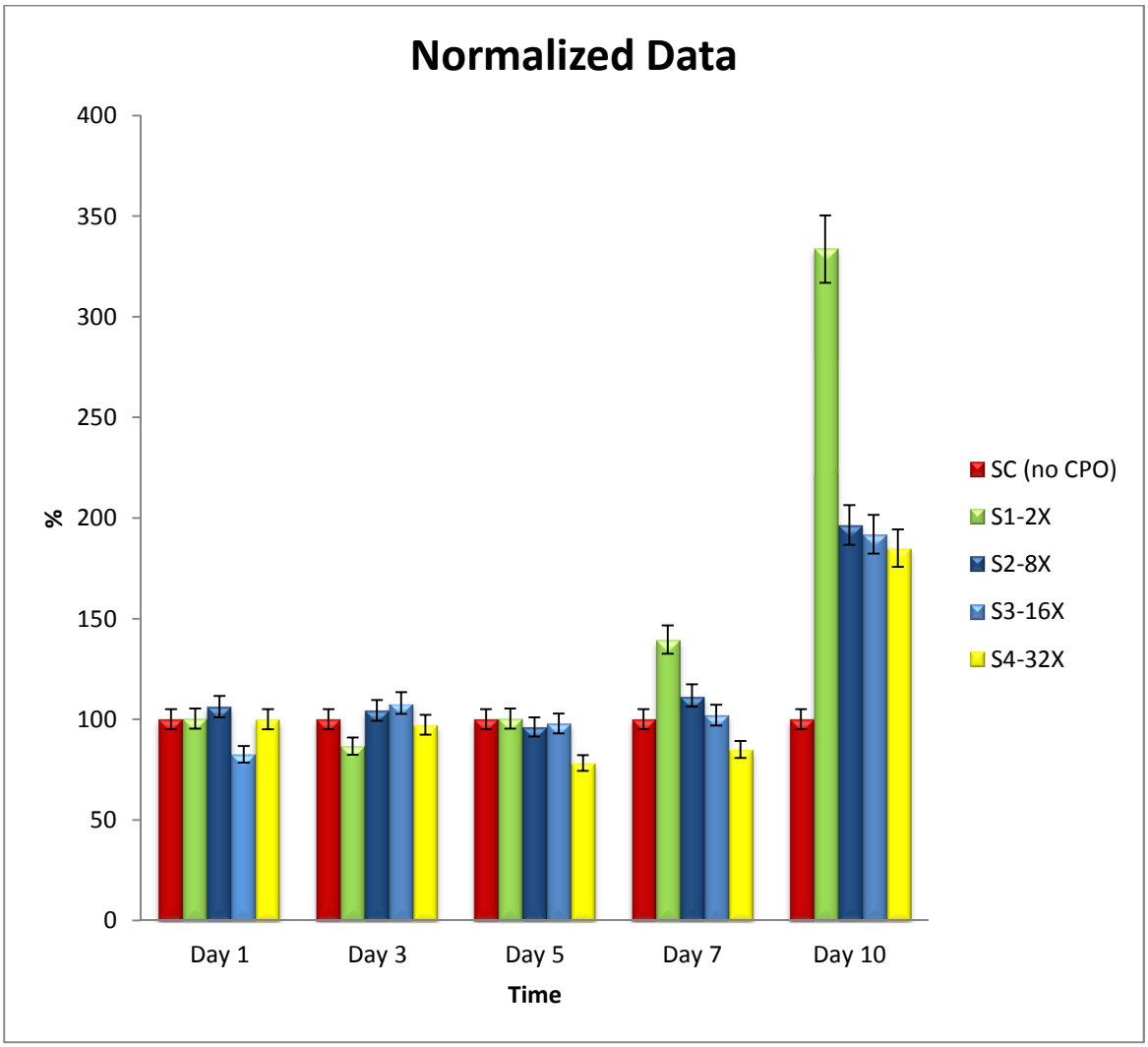

Figure 13. Normalized Plot with respect control (no CPO). The cells onto the S1-2X scaffold had 3.33 times more viability than control scaffolds at day 10 (P<0.05).

The figure 14 illustrates the cell morphology at day 10. The oxygenized scaffolds shown more cells attached to the hydrogel comparing with the control scaffold with no CPO. In the group of the oxygenized scaffolds the sample S1-2X shown more cell viability due to the oxygen diffusion rate was more adequate.

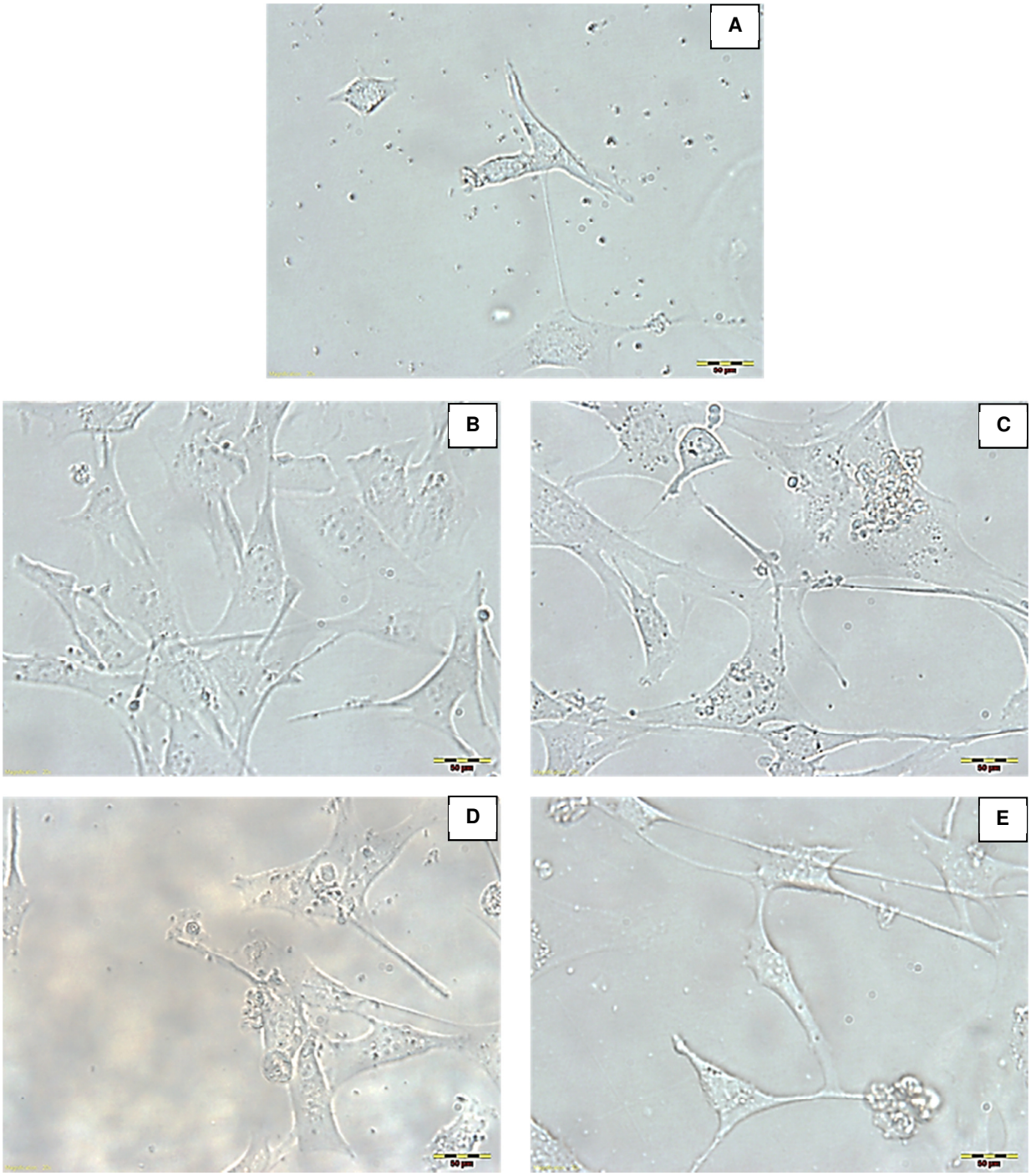

Figure 14. C2C12 cells morphology after 10 days under hypoxia conditions. (A) SC, control (no CPO), (B)S1-2X, (C) S2-8X, (D) S3-16X, (E) S4-32X.

6. CONCLUSIONS

6.1. Conclusions

In this research project, it has been demonstrated that this method can effectively deposit micromaterials at a level of nano-grams. Calcium peroxide has been used as an oxygen generating element capable of sustained and localized oxygen release when encapsulated within hydrogel (AA/$CaCl_2$). These scaffolds are able to provide adequate surroundings for cell survival and growth in hypoxic environments. We have shown that the oxygen released by these scaffolds is able to maintain cell viability within the construct under hypoxic conditions that mimic the in vivo tissue environment after implantation. The use of oxygen generating biomaterials may allow for increased cell survivability while neovascularization is being established after implantation. Such scaffolds may play an important role in tissue engineering where currently oxygen diffusion limits the engineering of large tissue implants.

The prevention of necrosis by the oxygen releasing biomaterial is a key finding of this study and suggests that the early supplementation with oxygen is able to delay tissue death.

6.2. Limitations – Further Place to Improvement

Work has to be done to design X-Y-Z platform along with the inkjet technology utilized in the study that can allow construct big and complex scaffolds. Further studies have to be done to evaluate oxygen release base in different hydrogel thicknesses. Current study was limited to evaluate single thickness.

The need of in vivo studies seems apparent, until now limited studies have been made evaluation oxygen releasing biomaterials in vitro sowing promising results. Therefore, it is expected that upcoming years more interest gets into evaluating such biomaterials.

REFERENCES

1. Folkman, J. and M. Hochberg, *Self-regulation of growth in three dimensions.* J Exp Med, 1973. 138(4): p. 745-53.

2. Khademhosseini, A., et al., *Microscale technologies for tissue engineering and biology.* Proc Natl Acad Sci U S A, 2006. 103(8): p. 2480-7.

3. Ahrendt, G., D. Chickering, and J. Pranieri, *Angiogenic growth factors: a review for tissue engineering.* Tissue Engineering 1998. 4(2): p. 117-130.

4. Smith, M.K., et al., *Locally enhanced angiogenesis promotes transplanted cell survival.* Tissue Engineering, 2004. 10(1-2): p. 63-71.

5. Atala, A., et al., *Tissue-engineered autologous bladders for patients needing cystoplasty.* Lancet, 2006. 367(9518): p. 1241-6.

6. Gordon, J.E., M.R. Dare, and A.F. Palmer, *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 Prog, 2005. 21(6): p. 1700-7.

7. Khattak, S.F., et al., *Enhancing oxygen tension and cellular function in alginate cell encapsulation devices through the use of perfluorocarbons.* Biotechnol Bioeng, 2007. 96(1): p. 156-66.

8. Oh, S.H., et al., *Oxygen generating scaffolds for enhancing engineered tissue survival.* Biomaterials, 2009. 30(5): p. 757-62.

9. Galwey, A. and W. Hood, *Thermal decomposition of sodium carbonate perhydrate in the solid state.* The Journal of Physical Chemistry, 1979. 83: p. 1810-5.

10. Cohen, I.S., *Regenerating the heart*. 2011, New York: Springer.

11. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. 275(5302): p. 964-7.

12. Schaper, W. and W.D. Ito, *Molecular mechanisms of coronary collateral vessel growth.* Circ Res, 1996. 79(5): p. 911-9.

13. Pepper, M.S., *Manipulating angiogenesis. From basic science to the bedside.* Arterioscler Thromb Vasc Biol, 1997. 17(4): p. 605-19.

14. Lévy, B.I. and A. Tedgui, *Biology of the arterial wall*. Basic science for the cardiologist. 1999, Dordrecht ; Boston: Kluwer Academic. xiv, 278 p.

15. Radisic, M., et al., *Biomimetic approach to cardiac tissue engineering: oxygen carriers and channeled scaffolds.* Tissue Eng, 2006. 12(8): p. 2077-91.

16. Leung R, Poncelet D, and N. RJ., *Enhancement of oxygen transfer rate using microencapsulated silicone oils as oxygen carriers.* J Chem. Technol Biotechnol, 1997. 68: p. 37-46.

17. Kim, H.W. and A.G. Greenburg, *Toward 21st century blood component replacement therapeutics: artificial oxygen carriers, platelet substitutes, recombinant clotting factors, and others.* Artif Cells Blood Substit Immobil Biotechnol, 2006. 34(6): p. 537-50.

18. Hanh, D.N., B.K. Rajbhandari, and A.P. Annachhatre, *Bioremediation of sediments from intensive aquaculture shrimp farms by using calcium peroxide as slow oxygen release agent.* Environmental Technology, 2005. 26(5): p. 581-589.

19. Baker, A.M. and W. Hatton, *Calcium Peroxide as a Seed Coating Material for Padi Rice .1. Requirement for and Provision of Oxygen.* Plant and Soil, 1987. 99(2-3): p. 357-363.

20. Ma, Y., et al., *Study on the generation mechanism of reactive oxygen species on calcium peroxide by chemiluminescence and UV-visible spectra.* Luminescence, 2007. 22(6): p. 575-580.

21. Harrison, B.S., et al., *Oxygen producing biomaterials for tissue regeneration.* Biomaterials, 2007. 28(31): p. 4628-34.

22. Painter, T., *Algal polysaccharides. In : Aspinall GO (ed) The polysaccharides .* Academic, New York, 1983: p. 196-286.

23. Govan, J.R., J.A. Fyfe, and T.R. Jarman, *Isolation of alginate-producing mutants of Pseudomonas fluorescens, Pseudomonas putida and Pseudomonas mendocina.* J Gen Microbiol, 1981. 125(1): p. 217-20.

24. Gorin, P.A.J. and J.F.T. Spencer, *Exocellular Alginic Acid from Azotobacter Vinelandii.* Canadian Journal of Chemistry, 1966. 44(9): p. 993-&.

25. Stanford, E., *On algin: a new substance obtained from some of the commoner species of marine algae.* Chem News, 1883. 96: p. 254-257.

26. Haug, A., B. Larsen, and O. Smidsrod, *Studies on Sequence of Uronic Acid Residues in Alginic Acid.* Acta Chemica Scandinavica, 1967. 21(3): p. 691-&.

27. Rehm, B., *Alginates : biology and applications*. Microbiology monographs,. 2009, Dordrecht ; New York: Springer. viii, 266 p.

28. Smidsrod, O. and G. Skjakbraek, *Alginate as Immobilization Matrix for Cells.* Trends in Biotechnology, 1990. 8(3): p. 71-78.

29. Winn, S.R., et al., *Behavioral Recovery Following Intrastriatal Implantation of Microencapsulated Pc12-Cells.* Experimental Neurology, 1991. 113(3): p. 322-329.

30. Soon-Shiong, P., et al., *Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation.* Lancet, 1994. 343(8903): p. 950-1.

31. Rokstad, A.M., et al., *Microencapsulation of cells producing therapeutic proteins: optimizing cell growth and secretion.* Cell Transplant, 2002. 11(4): p. 313-24.

32. Lim, F. and A.M. Sun, *Microencapsulated Islets as Bioartificial Endocrine Pancreas.* Science, 1980. 210(4472): p. 908-910.

33. Joki, T., et al., *Continuous release of endostatin from microencapsulated engineered cells for tumor therapy.* Nat Biotechnol, 2001. 19(1): p. 35-9.

34. Hasse, C., et al., *Parathyroid xenotransplantation without immunosuppression in experimental hypoparathyroidism: Long-term in vivo function following microencapsulation with a clinically suitable alginate.* World Journal of Surgery, 2000. 24(11): p. 1361-1366.

35. Lanza, R.P., R.S. Langer, and J. Vacanti, *Principles of tissue engineering*. 3rd ed. 2007, Amsterdam ; Boston: Elsevier / Academic Press. xxvii, 1307 p.

36. Kahn, T.J. and H. Ninomiya, *Changing vocabularies: a guide to help bioethics searchers find relevant literature in National Library of Medicine databases using the Medical Subject Headings (MeSH) indexing vocabulary.* Kennedy Inst Ethics J, 2003. 13(3): p. 275-311.

37. Xu, T., et al., *Inkjet printing of viable mammalian cells.* Biomaterials, 2005. 26(1): p. 93-9.

38. Xu, T., et al., *Viability and electrophysiology of neural cell structures generated by the inkjet printing method.* Biomaterials, 2006. 27(19): p. 3580-8.

39. Saunders, R.E., J.E. Gough, and B. Derby, *Delivery of human fibroblast cells by piezoelectric drop-on-demand inkjet printing.* Biomaterials, 2008. 29(2): p. 193-203.

40. Roth, E.A., et al., *Inkjet printing for high-throughput cell patterning.* Biomaterials, 2004. 25(17): p. 3707-15.

41. Miller, E.D., et al., *Dose-dependent cell growth in response to concentration modulated patterns of FGF-2 printed on fibrin.* Biomaterials, 2006. 27(10): p. 2213-21.

42. Ilkhanizadeh, S., A.I. Teixeira, and O. Hermanson, *Inkjet printing of macromolecules on hydrogels to steer neural stem cell differentiation.* Biomaterials, 2007. 28(27): p. 3936-43.

43. Barbulovic-Nad, I., et al., *Bio-microarray fabrication techniques--a review.* Crit Rev Biotechnol, 2006. 26(4): p. 237-59.

44. Yamazoe, H. and T. Tanabe, *Cell micropatterning on an albumin-based substrate using an inkjet printing technique.* J Biomed Mater Res A, 2009. 91(4): p. 1202-9.

45. Technologies. *M. Microdrop - Materials by Inkjet Technology*. 2010; Available from: http://www.microdrop.de/index.shtml?navid=1.

CURRICULUM VITA

Born in Ciudad Juárez, Chihuahua, México, March 23th, 1983, Daniel was the only son of Daniel Reyna Diaz and Maria Rosa Soriano Amaro. In the spring of 2006, he obtained his Bachelor of Science under the major of Electrical-Mechanical Engineering with a specialty in Advanced Manufacturing in his Hometown University, Technological Institute of Ciudad Juarez (Instituto Tecnológico de Ciudad Juárez). While pursuing his bachelor's degree, he starts working in January 2005 with DIMEYCO, a hometown mechanical engineering company, in the area of mechanical design; it stayed in the company remained after bachelor's graduation until August 2006; Few months after he obtained his Bachelor of Science degree, he starts working in August 2006 with Delphi Automotive Systems, a worldwide automotive company, reaching the position of Product Engineer. While full-time employed, in the spring of 2008, he enrolled into the adventure of pursuing the Master of Science degree in Mechanical Engineering at The University of Texas at El Paso. After serving for more than four years for the company he decided to fully devote his self into research in the emerging bioengineering area lead by Dr. Tao Xu at The University of Texas at El Paso. In 2010 he participates as a contest speaker during the AVS 57th International Symposium, and during the 5th Annual Research Colloquium organized by Texas Tech University Health Sciences Center in 2011.

Permanent Address:
Región de Bolonia 1301, Jardines de Santa Clara
Ciudad Juárez, Chihuahua, México 32563