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| Original Article |

Controlled Release of Oxygen from PLGA-Alginate Layered Matrix and its *In Vitro* Characterization on the Viability of Muscle Cells under Hypoxic Environment

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Abstract : Coagulative necrosis often occurs under hypoxic conditions, causing major limitation in the field of tissue engineering especially those dealing with larger tissues and organs. In this study, a comprehensive work has been performed in developing a tailor made design of a dual layered matrix that can produce oxygen to be utilized in tissue engineering application. Optimizations of protocol, ingredient and condition of the system were carried out specifically based on the responses observed from *in vitro* studies using L6 rat skeletal muscle cell as a candidate. Oxygen was generated from decomposition of encapsulated hydrogen peroxide. Poly (D,L)-lactide-co-glycolide (PLGA) with molecular weights of 90,000 and 110,000 $gmol^{-1}$ managed to secure good encapsulation of hydrogen peroxide for this application, while the best stirring time during the encapsulation was found to be 8 hours. The PLGA microspheres were coated with a secondary layer of alginate that was pre-grafted with calatase to form the dual layer system. This dual layered architecture has successfully controlled the release rate of oxygen at an optimum level for the survival of muscle cells under hypoxia condition. It was found that muscle cells have low tolerance limit towards the direct contact with hydrogen peroxide, however the cells maintained high viability within encapsulated hydrogen peroxide in the matrix system. It was observed that 4% of encapsulated hydrogen peroxide in the matrix system can produce efficient amount of oxygen at a controlled release manner to sustain the survival of muscle cells under hypoxic condition.

Key words: *controlled release of oxygen, alginate matrix, PLGA microspheres, muscle regeneration, hypoxia*

1. Introduction

Several tissue engineering approaches using cells are currently being investigated to repair, improve, or restore the function of damaged tissues.[1-3] However, a number of important issues need to be addressed to make these approaches a clinical success.[4] One major obstacle to the development of three dimensional tissue construct of clinically relevant sizes is the lack of a method to supply adequate nutrients and oxygen to the growing tissue in the period before it becomes fully vascularized *in vivo*. In addition, efficient removal of waste products during this period is important.[5] In normal human biological systems, blood transports nutrients and oxygen to cells. The cells in a tissue are located within the immediate vicinity of a blood vessel to ensure survival.[6] In most tissue engineering approaches, however, a tissue implant is usually developed *in vitro* and thus it has a low vascularization rate. Under such conditions, the distance between the cells and the sparse blood vessels with in these grafts is often on the order of multiple millimeters or even centimeters as the graft begins to grow even larger after implantation.[7] Consequently, limited oxygen and nutrient supplies and accumulation of waste products are likely to occur. Hypoxia commonly occurs with in the deeper regions of a constructas a result of the inadequate oxygen supply.[8, 9] In such case, regeneration of tissues of larger area through natural mean is often difficult.[10]

The limited supply of oxygen in tissue-engineered implants has attracted increasing attention because oxygen diffuses slowly through tissue and it has lower solubility and higher consumption rate compared to other major nutrients such as glucose.[9] To overcome this barrier, advancements in biomaterials research have led to the development of a control system that is capable

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of producing oxygen. With an oxygen-producing system, it is possible to delay the onset of hypoxia and to maintain cell viability and tissue structure.[11-13]

Various approaches to supply oxygen during tissue regeneration have been investigated. These include the use of oxygen rich fluids such as perfluorocarbons and silicone oil[13] in tissue perfusion. Other approaches involve the use of angiogenic factors, such as vascular endothelial growth factors (VEGFs), to promote vascularization within an engineered construct.[14] Although angiogenic response has been enhanced, the rate of angiogenesis cannot be accelerated,[12] and this limits the size of the three dimensional tissue masses that can be implanted for regenerative processes. Recently, a more straightforward method was reported by Harrison *et al*,[12] in which an oxygen generating salt was incorporated within the scaffolding used to construct tissue. This salt generated a continuous supply of oxygen to the growing cells over a period of time. However, controlled rates of oxygen and by-product formation generated by the decomposition of the salt remain major concerns.[15]

One approach for obtaining controlled release rates of various substances is to encapsulate the therapeutic agent within a polymeric matrix. The polymer acts as a rate-controlling membrane.[16, 17] Research of this area has been long performed by our group and recently we have successfully demonstrated the capability to encapsulated hydrogen peroxide (H_2O_2), a small aqueous-based molecule into a polymeric shell.[18] The study involved the direct encapsulation of H_2O_2 into a polymer matrix of poly (D, L)-lactide-co-glycolide (PLGA) via the double emulsion solvent evaporation method. The results demonstrated a successful encapsulation via a novel backward concentration gradient approach. As the H_2O_2 can be potentially decomposed into oxygen, our group pioneered in lifting a novel idea in this area of using pure encapsulated H_2O_2 system to generate clean oxygen for bio-medical applications.[19] The direct use of encapsulated H_2O_2 as oxygen source can eliminate the presence of side products such as metal cations as the decomposition produces only water and oxygen that are biologically safe. In order for the idea to work for an intended application, comprehensive study should be performed and optimization of the encapsulation parameters is needed from case to case basis. This is to ensure the system can perform at its optimum level as different cell lines will require different level of oxygen supply. Understanding this aspect, the work reported here was carried out to develop an oxygen generating system based on the same idea, with targeting specially toward muscle cell for tissue engineering application. The study will further investigate the response of the muscle cell sunder different conditions and concentrations of H_2O_2, besides demonstrating the best optimized oxygen generating system that can be utilized for muscle cells under stress of oxygen deficiency.

2. Materials and Methods

2.1 Chemicals and Reagents

All chemicals used are analytical or clinical grade unless otherwise stated. Poly (lactic-co-glycolic acid (PLGA) copolymers (50/50; Resomer RG 504H, Mw 55,000 $gmol^{-1}$ and RG 506, Mw 110,000 $gmol^{-1}$) and the 85/15 PLGA (Resomer RG 858S, Mw 220,000 $gmol^{-1}$) were purchased from Boheringer (Ingelheim, Germany). Polyvinyl alcohol (PVA, Mw=9000–11,000 $gmol^{-1}$), alginic acid sodium salt, calcium chloride dehydrate, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-Hydroxysuccinimide (NHS), hydrogen peroxide (H_2O_2, 50 wt %) and catalase were purchased from Sigma (St. Louis, MO, USA). Dichloromethane (DCM, Junsei Chemical, Japan) and triple distilled deionized water were used as solvents throughout the study. Media and reagents for cell culture were purchased from Gibco (Gibco BRL, Gaithersburg, MD, USA).

2.2 Encapsulation of H_2O_2 in PLGA

H_2O_2 loaded PLGA microspheres were prepared by the $W_1/O/W_2$ microencapsulation method as illustrated in Fig 1. Briefly, PLGA was dissolved in dichloromethane (DCM). Aqueous H_2O_2 was emulsified into the organic phase (O) of this preparation using a high speed homogenizer (Scientific Industries Inc., model G-560, USA) for 30 s to form the first W1/O emulsion. The resulting emulsion then was poured slowly into the aqueous phase which contained PVA and H_2O_2.

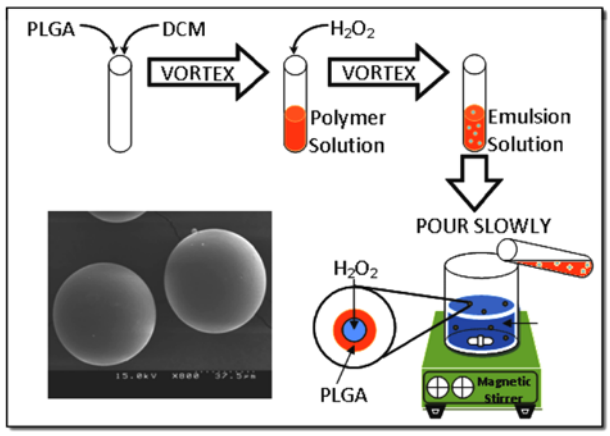

Figure 1. Illustration of experimental methodology employed for the synthesis of H_2O_2 encapsulated microspheres via double emulsion solvent evaporation method and insert showing SEM image of the microspheres.

This was followed by vigorous stirring using a magnetic bar. The pH was adjusted to 7.4 and the final emulsion was continuously stirred at ambient conditions for a minimum period of 4 hr to evaporate the DCM. The microspheres were sieved, collected by filtration, and washed several times with triple distilled deionized water. Finally, the collected microspheres were kept at -20°C when not in use to avoid evaporation or the decomposition of H_2O_2. Reference microspheres were produced using the same protocol. These were loaded with triple distilled deionized water without addition of H_2O_2.

2.3 Immobilization of Catalase

Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen. It catalyzes the decomposition of H_2O_2 into water and oxygen. In this experiment, catalase was added to the preparations to promote the decomposition of H_2O_2. In order to incorporate catalase into the system, direct immobilization onto the alginate chain was carried out using EDC/NHS (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide/ N-Hydroxysuccinimide) chemistry[20,21] Amide linkages are expected to form between the carboxylic group of the alginate and the amino group of catalase. Briefly, 50 mM of EDC was dissolved in 10 mL of a 3% alginate solution. 30 mM of NHS was added after 2 hr of gentle stirring and the mixture was continuous stirred for another 1 hr. 1.0 mL of catalase (3.0 wt%) was added drop wise into the mixture, which now contained alginate with activated carboxyl groups. The mixture was stirred again for 24 hr and then kept frozen (-20°C) while not in use.

2.4 Alginate Coating Layer

The diffusion of H_2O_2 out of the PLGA shell promotes the possibility of direct contact between intact H_2O_2 and the surroundings. Under normal conditions, the decomposition of H_2O_2 is reported to be rather slow, and thus it could have undesirable effects on the materials or tissues in the vicinity of its release. To avoid this, the H_2O_2- containing PLGA microspheres were coated with a layer of alginate that contained immobilized catalase. The production of this catalase-embedded alginate was in the previous section. This double layer design reduces the possibility that naked H_2O_2 will come into direct contact with the surroundings. The PLGA microspheres were coated with this alginate layer using a dripping method.[22, 23] Briefly, 20.0 mg of the PLGA microspheres was mixed with 1.0 mL of alginate-immobilized catalase. The mixture was then drawn into a syringe and slowly injected drop-wise into a solution of $CaCl_2$ (5%). The mixture was gently stirred for 10 min to allow the alginate to gel around the microspheres through complexation between the alginate chains and the Ca^{2+} ions. The microbeads were collected using a sieve and washed continuously with deionized water for several cycles. The microbeads were frozen at -20°C and stored for future use.

2.5 Oxygen Release Profile

Dissolved oxygen (DO) concentration was recorded using a portable oxygen meter (Orion Series 3-Star Benchtop, Thermo Fisher Scientific Inc, USA). The electrode of the meter (Orion 081010, Thermo Fisher Scientific Inc, USA) was attached to a commercial 10 mL borosilicate vial. This allowed the measurements of DO to be carried out in a closed system to avoid interferences from the oxygen in the environment. All measurements were carried out under biosafety cabinet. A fixed amount of microcapsules (30 mg) was incubated in 5 mL of deionized water within a sealed 10 mL borosilicate vial. After a predetermined duration of incubation, the cap of the vial was removed and replaced with the electrode for the DO measurement. After each measurement, the vial was resealed for the next measurement. All measurements were performed in triplicate. In order to minimize the fluctuation in the readings caused by environmental and instrumentation factors, readings for pure deionized water in the absence of microspheres were taken as a reference and all subsequent readings of samples were corrected against the reference readings.

2.6 Cell Culture

L6 rat skeletal muscle cells (American Type Culture Collection, ATCC) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% FBS (Gibco), 500 U/mL penicillin (Gibco), and 500 μg/mL streptomycin (Gibco). Confluent cells were treated with 0.25% trypsin/EDTA (Invitrogen, Karlsruhe, Germany) and centrifuged at 1200 x *g* for 5 min to harvest the cells. The cell number in the resulting single cell suspension was determined using a hemocytometer (DHC-N01, SKC Inc, GA, USA). Aliquots containing 2×10^4 cells/mL were added to each sample and cultured for 24 hr.

2.7 Normoxia and Hypoxia Conditions

Cell viability was assessed using the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] assay described in the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). This assay is based on the reduction of WST-8 into formazan dye by active mitochondria. Briefly, L6 muscle cells were cultured for 48 hr with 200 μg of either H_2O_2- containing PLGA microspheres or alginate-coated PLGA microspheres in 1.0 mL of DMEM. An

initial cell density of 2×10^4 cells/mL was used for normoxic as well as hypoxic conditions. To perform the assay, 1/20 volume of WST-8 solution was added to each well of a microtiter plate and incubated for 4 hr. 200 μL of supernatant was transferred into 96 wells plate and the absorbance at 450 nm was recorded using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Hypoxic conditions were created by flushing a Billups-Rothenberg modular incubation chamber (model MIC-101, Billups-Rothenberg, Del Mar, CA, USA) with a mixture of hypoxic gases. Subsequently, the chamber was charged with a gas mixture containing 1% O_2, 5% CO_2, and 94% N_2. Samples were sealed before placement in the tissue culture incubator. Gas phase can be achieved within 10 min after opening and closing of the inlets and outlets.

2.8 Statistics

Results are reported mean±standard deviation. Data analysis was performed using SigmaPlot version 11.0 (Systat Software, Inc., Point, Richmond, CA, USA) and Microsoft Excel 2003. To determine differences between time points for each formulation, a one-way analysis of variance (ANOVA) performed to determine statistical significance.

3. Results and Discussion

3.1 Optimization of PLGA for Microencapsulation

Choosing a suitable polymer for use in microencapsulation via the double emulsion solvent evaporation method is a crucial factor of success. The molecular weight and co-polymer ratio of the chosen polymer will control the release kinetics of H_2O_2 from the encapsulation shell. Thisinfluences the amount of oxygen generated by controlling how much diffused H_2O_2 reaches the catalase immobilized in the outer alginate layer. In this study, PLGA was employed as the main encapsulation matrix as it is safe and approved by the Food and Drug Administration (FDA) to be used in humans.[24] Optimization of the release profile of oxygen from the composite microspheres was achieved by controlling the properties of the PLGA used. From this study, the average size of the PLGA microspheres obtained via the double emulsion solvent evaporation method was of 60-100 mm.

This study has investigated the effect of PLGA molecular weight toward the release profiles of DO based on three chosen weights of 55,000, 90,000 and 110,000 g mol^{-1}. The results obtained (Fig 2) indicatethat molecular weight of PLGA do significantly affecting the release of DO. In general, low molecular weight PLGA (55,000 g mol^{-1}) provided a “burst” effect that caused drastic increase in dissolved oxygen concentration during the first few hours of incubation. This was suspected to be caused by more porous shell as a general trend of using lower molecular weight polymer for encapsulation. In addition, the microspheres could be suffering from mechanically unstable condition due to non-homogenized surface and this promotes diffusion rate of H_2O_2 out from the shell. The muscle cells might not able to sustain its survival under such high concentration of diffused H_2O_2. As the molecular weight was increased, the release of DO showed an increment on a prolonged period for more than 24 hr. This is postulated to be the effect of controlled release and demonstrated the potential of the material in supplying oxygen over a longer duration. Higher molecular weight demonstrated the ability in capturing the H_2O_2 better and causes slower diffusion, thus maintaining the DO level for longer time. This is useful especially for muscle cell as it consumes high amount of oxygen during proliferation, while vascularization needs sufficiently some time to occur.

In this study, all the three co-polymers having the LA:GA ratio of 50:50 was chosen due to its intermediate physical property.[25] Higher ratio of LA is found to increase the hydrophobic nature of the polymer, which directly reduces the encapsulation efficiency as H_2O_2 is of hydrophilic nature. On the other hand, lower ratio of LA may causes difficulties in microspheres producing process due to the poor solubility of the polymer in organic solvent. Based on this information and the obtained trend on the effect of molecular weight, another class of PLGA having the molecular weight of 220,000 g mol^{-1}, and the LA:GA ratio of 85:15 was tested. This class of PLGA was chosen to validate the assumptions that that higher molecular weight and LA ratio can give higher encapsulation efficiency. The result was plotted together in Fig 2 for comparison of general trend with the previously tested sample. During the first three hours of incubation, a steady increment of DO has been obtained and

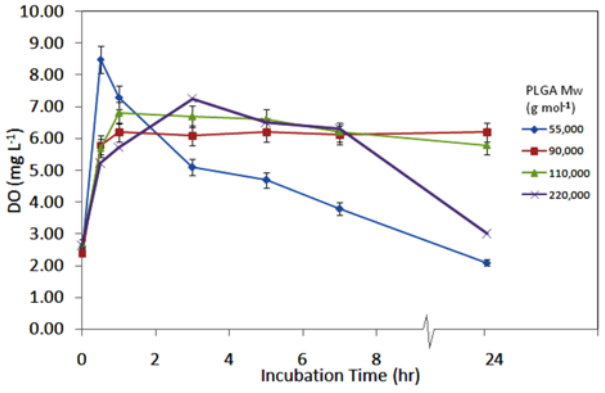

Figure 2. Effect of PLGA having molecular weight (a) 55000 (b) 90000, (c) 110000, and (d) 220000 g mol^{-1} on the release profile of DO. Results are presented as mean±standard deviation for n=3.

reached a considerably high level, agreeing with the assumption of better encapsulation efficiency. However, the result obtained showed a drastic drop after 7 hr of incubation and failed to maintain the DO level to the end of the study after 24 hr. This may be caused by the swelling of polymer as longer chain structure poses higher possibility of having loosened ends of the outer surface of the shell. These terminals can repel easily causing drastic swelling and distortion of the surface morphology, subsequently increasing the diffusion rate of H_2O_2. Therefore higher level of DO will be observed at the beginning, but could not sustains longer release period. Another factor contributed to this trend may be due to the higher hydrophilic nature of the microspheres having LA ratio of 85%. Diffusion rate will be theoretically higher as the matrix was more accessible by aqueous media, while H_2O_2 is of hydrophilic nature as well.

For the following studies, microspheres produced from intermediate molecular weight of 110,000 with LA:GA ratio of 50:50 was chosen. Theseparameters produce microspheres having denser shell for higher encapsulation efficiency, while manage to sustain oxygen level for longer duration. Tissue formation usually involves larger surface area and oxygen needs to be continuously supplied for a period of time before vascularization takes places.

3.2 Effect of Encapsulation Stirring Time

This work emphasizes the use of the double emulsion solvent evaporation technique to microencapsulate H_2O_2 and later utilizing the microspheres to facilitate cell survival. The method used to add H_2O_2 as well as the amount of this compound in the secondary stirring solution during microsphere formation were found to be crucial factors for effective microencapsulation of H_2O_2. We also found that time of stirring during the hardening stage was also effecting the final encapsulation efficiency. Fig 3 shows the oxygen release profile for microspheres produced with varying stirring times during the removal of organic solvent. The encapsulation efficiency of H_2O_2 was not significantly different for those microspheres prepared using the standard protocol with stirring times of more than 24 hr. This may be due to the high diffusion rate of small H_2O_2 out from the polymer matrix during the hardening stage. Measures have been taken to avoid this by reducing the stirring time to 8 hrs under constant air movement to reduce the diffusion of H_2O_2 out from the PLGA.

3.3 Effect of Alginate Coating Layer

H_2O_2 is a well known antiseptic for wound treatment. Although H_2O_2 is a simple molecule, it can be used to generate oxygen as it decomposes and this process produces no toxic

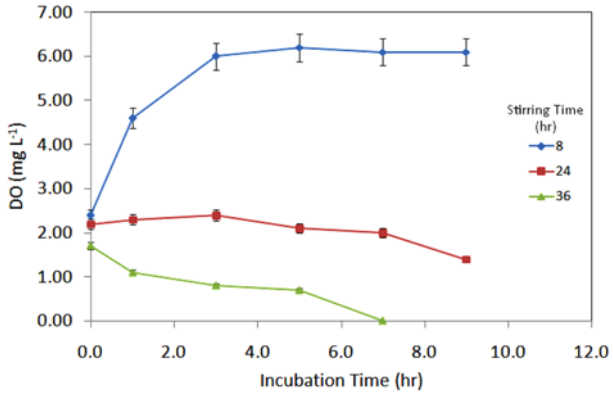

Figure 3. DO concentration for incubation solution after stirred for 8, 24, and 36 hr with the microspheres. Results are presented as mean±standard deviation for n=3.

products. However, if it is intended to be applied into muscle cell system, naked H_2O_2 might eventually cause harmful and toxic effect as muscle cells, usually like other living cells has low tolerance limit toward the direct contact with H_2O_2. This is because H_2O_2 is a strong oxidizing agent. If only a single layer encapsulation technique is used, there is of high possibility that naked H_2O_2 could diffuse into direct contact with cells. The decomposition of H_2O_2 is reported to be very slow in the absence of catalyst and this would also be problematic. In order to overcome these hurdles, further modifications of the microspheres might be an appropriate preventative method as reported in our recent rapid communication.[19] These issues have driven additional experiments in which a catalase-based, accelerated decomposition mechanism has been incorporated into the microsphere system. There are many types of matrices, especially polymer based materials, to which catalase can be covalently attached. In this study, alginate was used for this purpose. This selection was based on the facts that alginate is biocompatible, biodegradable, and water soluble. In addition, it can be obtained easily and is cost effective. Alginate chains possess carboxylic groups for use in attachment of secondary molecules, and their solubility can be changed using coordination chemistry. The average size of the PLGA microspheres coated with alginate that is pre-immobilized with catalase was of 1.0-2.5 mm. Fig 4 shows the morphological changes that occurred in PLGA microspheres when they were coated with an alginate layer after 24 and 48 hr.

The alginate coating provides another barrier between cells and the H_2O_2. Once the H_2O_2 has diffused out of the PLGA microspheres, decomposition of H_2O_2 occurs in the catalase-alginate layer to form O_2 and water. Thus, by varying the concentration of alginate used to coat the microspheres, the

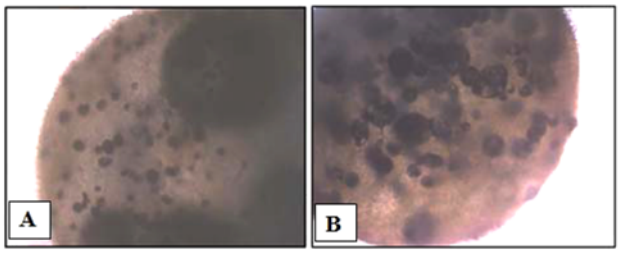

Figure 4. Morphological change of PLGA microspheres coated with alginate immobilized with catalase after (A) 24 hr and (B) 48 hr of incubation.

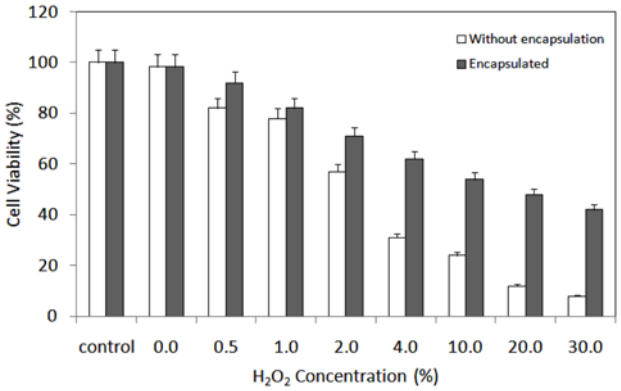

Figure 5. Comparison of naked and encapsulated H_2O_2 in single PLGA shell on the viability of the skeletal muscle cells at different concentrations after 48 hr under normoxia condition. Results are presented as mean±standard deviation for n=3.

release rate of oxygen can be controlled as mentioned previously.[19] The same general trend of DO release was observed in this study, which lower concentrations of alginate showed higher release rates at the beginning of the experiment but the rate dropped after 6 hr. This is likely due to the high porosity of the alginate layer that results when the concentration is not high enough to produce a closely packed layer. As a result, oxygen was released quickly, but this release rate could not be sustained for long time periods as the source of H_2O_2 runs low. Conversely, increasing the concentration of alginate can produce a more tightly packed coating, resulting in slower diffusion and increased release times.

3.4 Effect of Microencapsulation

If the microspheres shell could be designed for controlled release and complete decomposition, then it would be possible to use H_2O_2 for generating oxygen in tissue engineering application. In order to demonstrate the significance of the layered design, cell viability studies on the muscle cells have been performed. However quantitative assessment of viable cells under acidic circumstances caused by, e.g., normoxia and hypoxia significantly affect outcomes of the assay.[26] Therefore large amount of media were used for the assessment of viable cells under various experimental situations in these studies to reduce the effect of pH. Fig 5 shows the viability of L6 rat skeletal muscle cells that were exposed to naked H_2O_2 or encapsulated H_2O_2 of different concentrations. The initial study was performed using only single layered microspheres, which having only PLGA shell without the alginate layer. The purpose of this study was to obtain evidence on the significanceof polymeric encapsulation compared to the one with direct contact with H_2O_2. This study was important as different cell lines will have different tolerance limits and thus it should be identified for each system to be applied. Optimization on multi-layer coatings for better control release will be demonstrated in later section, using alginate layer. As expected, direct contact of H_2O_2 with the muscle cells resulted in toxicity and a reduction in the cell viability. However, when encapsulated H_2O_2 was used, the viability of the cells was increased, and this was most likely due to the controlled release of H_2O_2 and the barrier layer between the cells and H_2O_2. In general, higher concentration of H_2O_2 led to more toxicity, and at all concentrations, encapsulated groups demonstrated significantly increased viability compared to naked H_2O_2. This proved that encapsulation can provide good alternative in reducing the H_2O_2 toxicity on muscle cells, while it is believed that better outcome can be obtained with further optimizations and modifications.

3.5 Oxygen Release Profile in Hypoxia Condition

The key objective of this study was to attempt to prevent tissue necrosis for muscle cell using an oxygen-releasing biomaterial. It has been suggested that the early supplementation with oxygen can delay tissue death. It has also been reported that normal degradation processes associated with hypoxia (skin discoloration, increased apoptosis, and lactate buildup) can be slowed in the presence of oxygen, but the degradation process resumes when the oxygen is exhausted. Using oxygen producing biomaterials, we have decreased the onset of necrosis and maintained cell viability and tissue structure in critically perfused tissue. While oxygen generation can occur over a period of hours, it is realistic to believe that with further optimization, oxygen production can be sustained for days. During this time, growth factors can be used to support the enhanced formation of new vesselsin the engineered tissue. Since engineered tissue of clinically relevant size will require oxygen to sustain viability until the supporting vascular is formed, our approach was to supply oxygen during this critical time. To mimic this in vitro, a

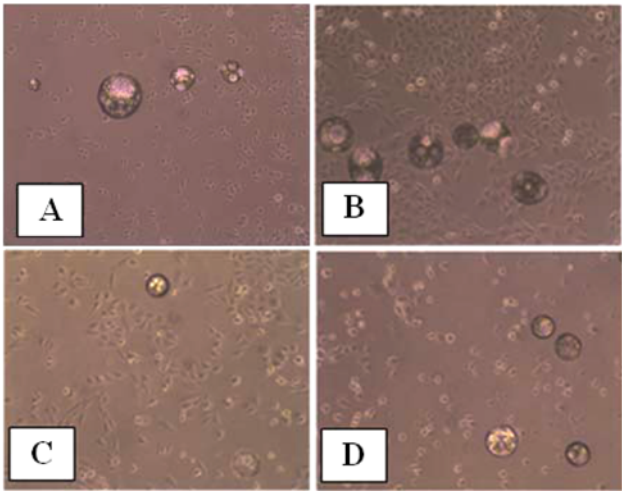

Figure 6. Microscopic images of skeletal muscle cells taken after incubated for 48 hr under hypoxia condition treated with the H_2O_2 encapsulated in PLGA microspheres having (A) 0% (B)4% (C) 10% and (D) 20% of H_2O_2.

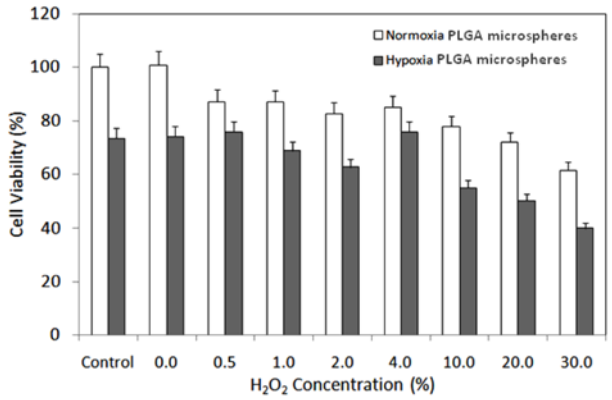

Figure 7. Viability assay of the skeletal muscle cells incubated with different concentrations of H_2O_2PLGA microspheres after 48 hr under normoxia and hypoxia conditions. Results are presented as mean±standard deviation for n=3.

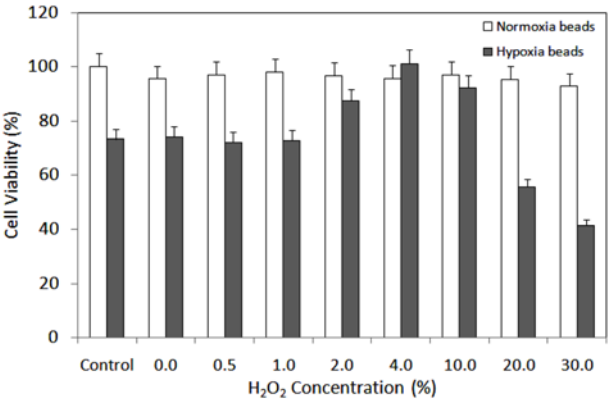

Figure 8. Viability assay of the skeletal muscle cells after 48 hrsculture under normoxia and hypoxia conditions incubated with H_2O_2PLGA microspheres coated with alginate layer. Results are presented as mean±standard deviation for n=3. The 4% sample showed a significantlyhigher cell viability under hypoxic condition when compared to the control group ($p < 0.001$).

new study was carried out using microspheres encapsulated in PLGA without the alginate layer. Fig 6 shows the microscopic images of skeletal muscle cells exposed to different concentrations of encapsulated H_2O_2 under hypoxic conditions. It was observed that at 4% encapsulated H_2O_2 increased viability of cells even under hypoxia. This important evidence indicated that the biomaterials developed in this study can provide sufficient oxygen to muscle cells that were set to be under hypoxiaenvironment. However, higher concentrations of encapsulated H_2O_2 resultedin causing toxicity effect, which indicate non-optimal condition for complete decomposition of H_2O_2.

Fig 7 shows the viability of the cells exposed to various concentrations of H_2O_2 in both normoxia and hypoxia. PLGA microspheres containing 10%, 20% and 30% H_2O_2 caused higher toxicity in these cells compared to lower concentrations (0.5%, 1%, 2% and 4%). Significant decreases were observed in the proliferation of skeletal muscle cells as the concentration of H_2O_2 is higher than 10% in both normoxia and hypoxia environment.

It is well known that the viability of SMCs is lost when there is a prolonged interruption of blood supply *in vivo*. Lacking of oxygenresults in ischemia, and decrease inskeletal muscle function. However by coating the PLGA microspheres with alginate that is pre-immobilized with catalase, these experiments have shown that oxygen producing microspheres maintain SMCs viability undersuch hypoxic conditions (Fig 8). The muscle cells incubated with the microspheres containing an alginate coated layer were observed to be healthy and these cells could proliferate. Fig 8 shows the growth and viability of SMCs cultured under different concentrations of oxygen. Control cultures were grown under the same conditions without microspheres. The viability of SMCs was observed to be significantly higher in the cultures containing microspheres with 4% encapsulated H_2O_2 under hypoxia. However lower concentration of encapsulated H_2O_2 i.e. 0.5, 1, and 2 % did not have any significant effect on the cellular activity of SMCs. As expected, higher concentrations of encapsulated H_2O_2 decrease the proliferation and viability of the SMCs.

4. Conclusion

One of the major problems in scaffold based tissue engineering is the occurrence of tissue quality gradients within 3D tissue constructs. These tissue gradients are formed when nutrient and oxygen supply are limited within the 3D environment, and this can occur even if dynamic culture systems are used. In this study, oxygen producing microspheres have been constructed, and it has been demonstrated that these are capable of increasing cell viability under hypoxic conditions. Sustained release of oxygen from decomposition of encapsulated H_2O_2 using catalase embedded in an alginate layer was observed. The results have demonstrated that oxygen producing microspheres can provide an adequate environment for cells to overcome the hypoxia condition. These oxygen producing microspheres may play an important role in scaffold based tissue engineering because they provide a solution towardsoxygen diffusion limitation in the engineering of large tissue implants.

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