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Oxygen-releasing biomaterials for tissue engineering

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Abstract

Due to the increasing demand to generate thick and vascularized tissue-engineered constructs, novel strategies are currently being developed. An emerging example is the generation of oxygen-releasing biomaterials to tackle mass transport and diffusion limitations within engineered tissue constructs. Biomaterials containing oxygen-releasing molecules can be fabricated in various forms, such as hybrid thin films, microparticles or three dimensional scaffolds. In this perspective, we summarize various oxygen-releasing reagents and their potential applications in regenerative engineering. Moreover, we review the main approaches for fabricating oxygen-releasing biomaterials for a range of tissue engineering applications.

Keywords: oxygen-releasing biomaterials; peroxides; cell survival; tissue engineering

INTRODUCTION

Every year millions of people suffer from loss of life or other health-related issues associated with organ failure.[1] Due to limitations with the number of available organ donors, there is an increasing demand for artificial tissue replacements. Tissue engineering aims to repair diseased or damaged tissues to improve human health.[2] A crucial requirement for the fabrication of vascularized thick tissue constructs is to provide sufficient oxygen for the metabolically active cells encapsulated within the three dimensional (3D) structure of the engineered scaffolds. The presence of oxygen facilitates graft maturation especially during the early stages of tissue formation.

The dimensions of tissue-engineered constructs should comply with mass transport and diffusion limits to yield viable tissue constructs. For example, when the tissue thickness exceeds 1 mm, due to the limited oxygen diffusion into the 3D matrix, hypoxia conditions establish within the microenvironment as a function of time. This results in reduced cell viability within the 3D constructs. To tackle this hurdle, there have been recent efforts focusing on the generation of oxygen-releasing biomaterials as transplantable constructs.[3,4]

Inadequate levels of oxygen have been shown to induce apoptosis and necrosis within 3D tissues both *in vitro* and *in vivo*.[5–8] The use of oxygen-releasing constructs could decrease apoptosis and necrosis by providing sufficient oxygen for an extended period of time.[3,4] Thus, it may be of benefit to utilize these materials during maturation of newly formed functional tissues. Another potential application for oxygen-releasing biomaterials is for the treatment of ischemic tissues, such as cardiac muscle after myocardial infarction. Moreover, these biomaterials have great potential to promote healing of large and chronic wounds. However, one common problem with oxygen-delivering materials is the sudden release of oxygen, which may damage cells. Therefore, it is essential to design biomaterials with sustained oxygen-release capabilities for tissue engineering applications. The release kinetics of an ideal oxygen-releasing biopolymer should be tunable and extended from days up to weeks to allow sufficient time for revascularization and maturation of the engineered graft within the host system.[9]

The most common oxygen-releasing materials include sodium percarbonate,[4] calcium peroxide,[3,9] magnesium peroxide,[4] hydrogen peroxide[10,11] and fluorinated compounds.[12–14] Solid peroxides decompose upon exposure to water to release oxygen. However, if this process takes place too quickly, it may significantly damage cells due to formation of free radicals.[15] The rate of oxygen release via peroxide compounds is influenced by a number of factors such as temperature, pH and presence of a buffer or catalyst.[16,17] For example, when solid peroxide compounds react with water they form metal hydroxides, which induce an increase in the pH and the amount of released oxygen.[18] Alternatively, the use of buffers may provide adjustment in the pH and therefore oxygen generation. Moreover, the purity and solubility of peroxides significantly affect the kinetics of oxygen release.

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Oxygen-releasing biomaterials can be produced in various forms including thin films, microparticles, 3D scaffolds or cell-laden hydrogels. The use of these biomaterials improves cell survival under hypoxic conditions, where oxygen supply is limited. In addition, oxygen-producing biomaterials can potentially enhance vascularization and angiogenesis events. This is crucial for a wide range of applications in regenerative engineering, especially for cardiac and pancreatic tissue formation and wound healing processes.

OXYGEN-GENERATING MATERIALS

Solid inorganic peroxides such as calcium peroxide (CaO_2), sodium percarbonate ($(Na_2CO_3)_2{\cdot}1.5H_2O_2$) and magnesium peroxide (MgO_2) have been proposed for generation of oxygen within liquid environments. As described in the chemical reactions below, formation of hydrogen peroxide (H_2O_2) takes place in the first step upon exposure of solid inorganic peroxides to water (Eqns (1)–(3)). This is subsequently followed by decomposition of H_2O_2 into oxygen in the second step (Eqn (4)):[17,18]

$$CaO_2\,(s) + 2H_2O \rightarrow Ca\,(OH)_2\,(s) + H_2O_2 \quad (1)$$

$$MgO_2\,(s) + 2H_2O \rightarrow Mg\,(OH)_2\,(s) + H_2O_2 \quad (2)$$

$$(Na_2CO_3)_2 \cdot 3H_2O_2 \rightarrow 4Na^+ + 2CO_3^-2 + 3_H2O_2 \quad (3)$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (4)$$

MgO_2 allows for the slowest oxygen formation compared to the other solid reagents given above due to its lower solubility. For example, it was found that the equilibrium coefficients for MgO_2 and CaO_2 are 1.8×10^{-11} and 9.8×10^{-9}, respectively[18] indicating the lower solubility and consequently slower reaction rate for MgO_2 compared to CaO_2 with water.[19] Moreover, the availability of higher purity commercial formulations of CaO_2 enables more efficient delivery of oxygen compared to MgO_2.[20] For instance, CaO_2 can be found with 60–80% purity, whereas MgO_2 can only be obtained at *ca* 15–25% purity by weight.[21] Therefore, CaO_2 is commonly preferred as a reagent among the other solid peroxides. In addition, CaO_2 is an inexpensive reagent and has a long history of applications as an oxygen-releasing compound. All of the above solid inorganic peroxides have been used in tissue engineering as oxygen-releasing reagents due to their cellular compatibility.[3,4,22]

The rate of oxygen release is of significant importance for tissue construct formation. For example, if oxygen release takes place too quickly, the oxygen cannot be utilized due to supersaturation. On the other hand, if the oxygen is released too slowly, it does not provide a sufficient source to maintain healthy cellular function. Therefore, the ability to provide oxygen in a controlled and sustained manner may have important implications for biological systems. The rate of oxygen formation from peroxide compounds depends on a number of factors, including temperature, pH, ratio of solid peroxide to water, amount of catalyst and type of catalyst.[23,24] In addition, the hydrophilicity of the surrounding biopolymer also influences the release rate of oxygen from the source. For example, if a hydrophobic material is used to encapsulate solid peroxides, the rate of oxygen release reaction is slow due to the slow diffusion of water into the hydrophobic materials.[9] In this case, solid peroxide particles do not immediately come into contact with water, which leads to a slow release of oxygen. On the other hand, in the case of hydrophilic materials, water adsorption happens quickly and thus solid peroxide particles start decomposing and generating oxygen faster. Both hydrophilic and hydrophobic oxygen-releasing biomaterials have been used for various tissue engineering applications.[3,4,22]

Catalase is often used as a catalyst for facilitating the conversion of H_2O_2 into oxygen.[25] Catalase is an enzyme, present in the liver and blood of mammals, and is used to decompose H_2O_2 into water and oxygen[16] with very high turnover efficiency.[26] This enzyme is composed of four heme (iron-containing organic ring) groups embedded within its structure to be utilized in oxygen-conversion processes. Although the exact mechanism of the catalase function is unknown, it is believed that the mechanism of the decomposition reaction of H_2O_2 is given by the following equations:

$$H_2O_2\,(aq) + 2Fe^{3+}\,(aq) \rightarrow O_2\,(g) + 2Fe^{2+}\,(aq) + 2H^+\,(aq) \quad (5)$$

$$H_2O_2\,(aq) + 2Fe^{2+}\,(aq) + 2H^+\,(aq) \rightarrow 2H_2O\,(l) + 2Fe^{3+}\,(aq) \quad (6)$$

Without catalase, the formation of H_2O_2 may lead to unwanted side reactions and cellular damage. Therefore, using a catalyst is a common strategy for the conversion of H_2O_2.

In addition to the physical and chemical properties of biomaterials, the oxygen consumption rate is also influenced by cellular density and metabolism. For instance, hypoxia conditions are accelerated throughout a construct in the case of a high density of highly metabolically active cells.[9]

Although solid peroxides are commonly utilized as oxygen sources, liquid H_2O_2 can also be used for oxygen generation. In this case, only Eqn (4) takes place for the formation of oxygen.[15] It has been previously shown that CaO_2, $(Na_2CO_3)_2{\cdot}1.5H_2O_2$ and MgO_2 are more effective sources for *in situ* oxygen formation as compared to liquid H_2O_2.[16,19,27,28] This is because oxygen generation can be achieved in a more controlled and sustained manner by the use of solid peroxides, which is a highly desirable feature for tissue engineering research.

In addition to solid and liquid peroxides, it is also possible to use perfluorocarbons (PFCs) as oxygen supply for tissue engineering applications. PFCs have the ability to dissolve large amounts of physiologically important gases, such as oxygen and carbon dioxide. These fluorinated liquids could potentially be used for tissue engineering applications where there is a high demand for oxygen (i.e. hypoxia conditions).[13,29,30] PFCs can be used as aqueous emulsion systems[12] or can be embedded into a suitable biomaterial[14] to supply oxygen. In liquid emulsions, oxygen trapped in the core of particles can only be transferred by diffusion since PFC droplets have a higher density than and are immiscible with water.[13,14]

The amount of released oxygen is described as 'dissolved oxygen' in aqueous solutions and measured using different approaches such as with the use of oxygen sensors[4,9] or optical set-ups that utilize dye complexes.[31] To determine the kinetics of oxygen release, one common approach is to use electrochemical sensors that can reduce oxygen on a noble metal electrode, such as platinum.[32] In this method, dissolved oxygen is measured in the liquid medium in terms of ppm or mm Hg. However, due to their shortcomings, such as inaccurate measurements in low-oxygen environments, alternative methods have been developed. For instance, O'Neal *et al*. fabricated an optical system, where a ruthenium complex was used to detect the amount of oxygen in cell culture media.[31] In that study, the release kinetics of oxygen was determined based on the change in color intensity. Alternatively, oxygen concentration in liquid environments can be determined using the Trinder reaction.[22]

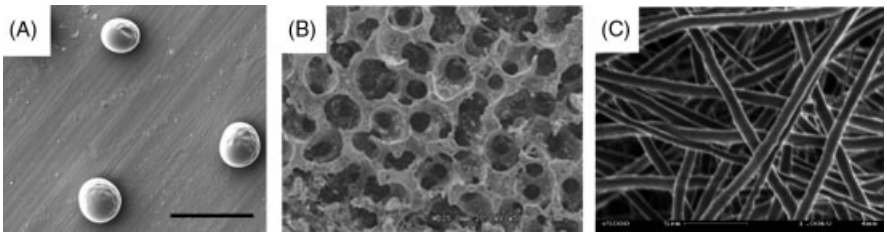

Figure 1. SEM images for solid peroxide-incorporated biomaterials. (A) Core–shell H_2O_2–poly(2-vinylpyrrolidone) microspheres. (Li *et al*.[15] Copyright 2012 with permission from Elsevier.) (B) 5% CaO_2–poly(D,L-lactide-*co*-glycolide) scaffolds. (Oh *et al*.[3] Copyright 2009 with permission from Elsevier.) (C) 10% CaO_2–polycaprolactone electrospun nanofibers. (Wang *et al*.[33] Copyright 2011 American Chemical Society.)

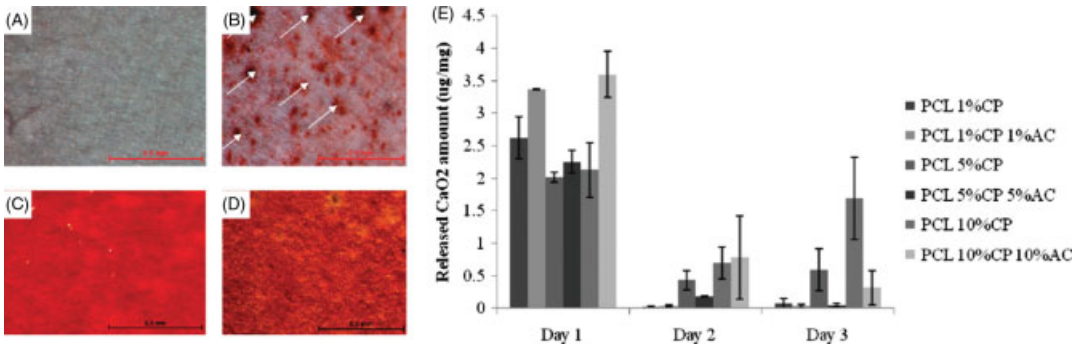

Figure 2. Characterization of CaO_2-incorporated PCL nanofibers. Alizarin red staining for calcium in (A) pure PCL nanofibers, (B) 1% CaO_2-blended PCL nanofibers, (C) 5% CaO_2-blended PCL nanofibers and (D) 10% CaO_2-blended PCL nanofibers. (E) Quantification of the amount of CaO_2 released from the nanofibers with and without ascorbic acid (AC), which enhanced the burst release. (Wang *et al*.[33] Copyright 2011 American Chemical Society.)

APPLICATIONS OF OXYGEN-RELEASING BIOMATERIALS IN TISSUE ENGINEERING

Numerous approaches have been developed to incorporate oxygen-releasing molecules into biomaterials for *in situ* generation of oxygen (Fig. 1). The most widely used methods are adsorption of oxygen-releasing molecules into fibers[33] or scaffolds,[3] encapsulating them within 3D polymer networks[15] and direct administration of oxygen-carrying reagents into a liquid medium.[10] Various assays have been used to test cellular response to oxygen-releasing biomaterials, such as cell viability, metabolic activity, proliferation and apoptosis assays (live/dead, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS), caspase activity, and lactate dehydrogenase).[3,4,9,15]

Solid peroxide compounds have been incorporated into electrospun nanofibers to form oxygen-releasing fibrous scaffolds for tissue engineering applications. For instance, in one report, CaO_2 particles were blended into polycaprolactone (PCL) at different concentrations (1, 5, 10% w/w) prior to electrospinning to fabricate hybrid nanofibers with or without the addition of a cytoprotective reagent, ascorbic acid.[33] The incorporation of CaO_2 into the PCL nanofibers was validated by alizarin red staining (Figs 2(A)–(D)). The release of oxygen was determined by incubating the nanofibers in deionized water for different time periods and testing the presence of calcium in the solution using a colorimetric assay (Fig. 2(E)). The burst release of CaO_2 was found to occur in day 1. The addition of ascorbic acid was determined to increase the burst release due to enhanced pore sizes. The evaluation of antibacterial properties of the oxygen-releasing nanofibers was determined by incubating them with *Staphylococcus epidermidis* and *Escherichia coli*. The results indicated that there was significantly less bacterial activity when CaO_2–PCL nanofibers were used. To test mammalian cell response against the CaO_2–PCL nanofibers, human osteoblast cells were seeded on the nanofibers and their proliferation was measured up to 4 days. CaO_2 cytotoxicity was reported to be significantly higher on day 1 compared to day 4, which was due to the burst release of oxygen at day 1. The CaO_2–PCL nanofibers could be used for preventing the colonization of bacteria on the surface of artificial prostheses and decrease the risks of infection. Although this strategy was shown to be potentially useful, sustained delivery of oxygen is crucial and needs to be addressed for numerous applications in tissue engineering.

Towards clinical translation, Harrison *et al*. encapsulated peroxide compounds within polymeric scaffolds to study cellular response under hypoxic conditions.[4] In that work, $(Na_2CO_3)_2{\cdot}1.5H_2O_2$-dispersed poly[lactic-*co*-(glycolic acid)] (PLGA) films were generated using a solvent-casting technique. The release of oxygen from the film was confirmed by observation of gas bubbles over 24 h. The release of oxygen slowed down and was complete by 70 h. The $(Na_2CO_3)_2{\cdot}1.5H_2O_2$–PLGA films were then implanted subcutaneously in a skin flap nude mouse model to determine the amount of necrosis at different time points up to 7 days. It was observed that the oxygen-releasing films significantly decreased *in vivo* necrosis and lactate levels, indicating the benefits of using oxygen supplementation in a wound healing model (Fig. 3). It should be noted that the generation of oxygen for prolonged periods of time is preferred for a wide range of tissue engineering applications. Therefore, it is essential to develop approaches that control sustained release of oxygen over longer periods of time. For example, encapsulation of $(Na_2CO_3)_2{\cdot}1.5H_2O_2$ within a more hydrophobic polymer could potentially address this issue. The key finding of the work was the reduced tissue death as a result of the use of oxygen-releasing films demonstrating the benefits of localized effect of oxygen delivery in ischemic tissues.

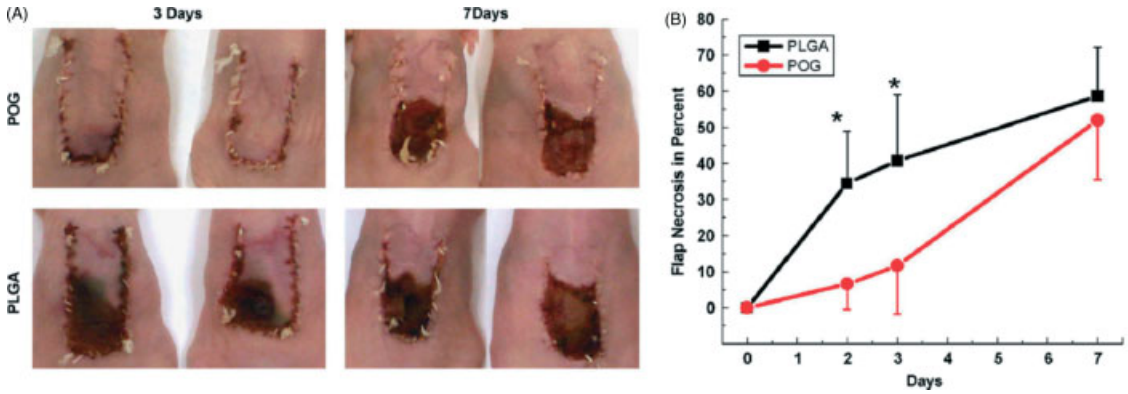

Figure 3. Polymeric oxygen-generating (POG) PLGA films for tissue regeneration. (A) *In vivo* model demonstrating flap necrosis. (B) Quantification of percent flap necrosis. (Harrison *et al*.[4] Copyright 2007 with permission from Elsevier.)

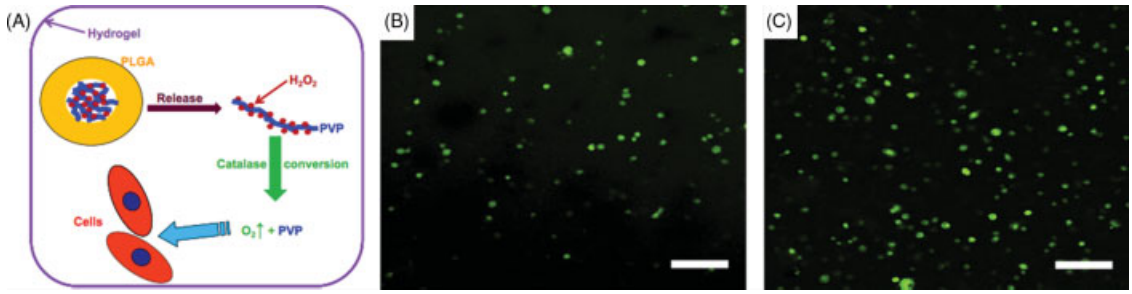

Figure 4. Oxygen-releasing H_2O_2–PVP–PLGA scaffolds to enhance CDC survival and differentiation upon exposure to 1% oxygen. (A) Schematic of the synthesis of H_2O_2–PVP–PLGA scaffolds. CDCs were encapsulated within a thermoresponsive hydrogel (B) without and (C) with oxygen-releasing H_2O_2–PVP–PLGA blend. The viability of CDCs was determined using a live/dead assay after 14 days of culture under hypoxic conditions. (Li *et al*.[15] Copyright 2012 with permission from Elsevier.)

In addition to solid peroxides, H_2O_2 in liquid form has been used in oxygen delivery studies. For example, Li *et al*. developed a method to fabricate core–shell oxygen-releasing microspheres for augmentation of cell survival under hypoxic conditions.[15] They first generated microspheres of H_2O_2-bound poly(2-vinylpyrrolidone) (PVP)-loaded PLGA using a coaxial device (Fig. 4). Then, the microspheres and cardiosphere-derived cells (CDCs) were encapsulated within a temperature-sensitive hydrogel system consisting of acrylic acid, *N*-isopropylacrylamide and hydroxyethyl methacrylate-oligo(hydroxybutyrate) to test the survival and differentiation ability of CDCs. The rate of release of oxygen was tunable by varying the ratio of H_2O_2 to PVP. Cell viability was around 57.3% when CDCs were exposed to hypoxia conditions within control hydrogels (without H_2O_2) at day 7. In addition, the differentiation of CDCs stopped upon exposure to hypoxia after one week. In contrast, the viability of CDCs was found to be significantly enhanced within the oxygen-releasing hydrogel system. Similarly, the differentiation capacity of CDCs was significantly augmented in the oxygen-delivering hydrogel. It was reported that this strategy enabled release of oxygen for up to two weeks. H_2O_2-releasing microspheres could be used for sustained delivery of oxygen to myocardial-infarcted tissues, which have been exposed to hypoxia during the early stages of the damage. This approach may also be of benefit for use in other cellular therapies where effective delivery of cells is required.

Oxygen-releasing materials are valuable for the formation of 3D scaffolds, which are more useful compared to two-dimensional techniques for various tissue engineering applications, such as vascularization. The generation of pre-vascularization within engineered scaffolds could address the issues relevant to adequate delivery of oxygen. In this context, incorporation of oxygen-releasing molecules within engineered tissues may provide additional oxygen to the construct and avoid the problems caused by lack of oxygen, such as tissue necrosis. To test this hypothesis, Oh *et al*. encapsulated solid CaO_2 particles within 3D PLGA using a porogen leaching procedure to enable the release of oxygen for at least 10 days[3] (Fig. 1(B)). The release of oxygen from the scaffold was determined by incubating the scaffolds in serum-free medium in a glovebox under hypoxia conditions (1% oxygen) and daily measurement of the dissolved oxygen in the medium was carried out by using a gas analyzer during 10 days. The amount of oxygen in the medium was found to be significantly higher when compared to the PLGA scaffold without CaO_2 particles. To test the cellular response to oxygen-releasing material, 3T3 fibroblast cells were seeded on the PLGA scaffolds and incubated under hypoxia conditions. Cellular viability and growth were tested using a standard MTS assay at days 1, 3, 5, 7 and 10. Cellular activity was observed to be significantly decreased after 3 days in the control scaffolds without CaO_2 particles, whereas the oxygen-releasing scaffolds exhibited an increasing trend for metabolic activity until day 10. The oxygen-releasing scaffold maintained significantly higher levels of oxygen under hypoxic conditions compared to control samples of plain PLGA scaffolds. Similarly, cellular viability was found to be improved for the oxygen-releasing scaffolds. The oxygen-releasing scaffolds fabricated in the study cited could be useful as transplantable constructs *in vivo* and are expected to maintain viable tissue constructs until formation of vascular networks.

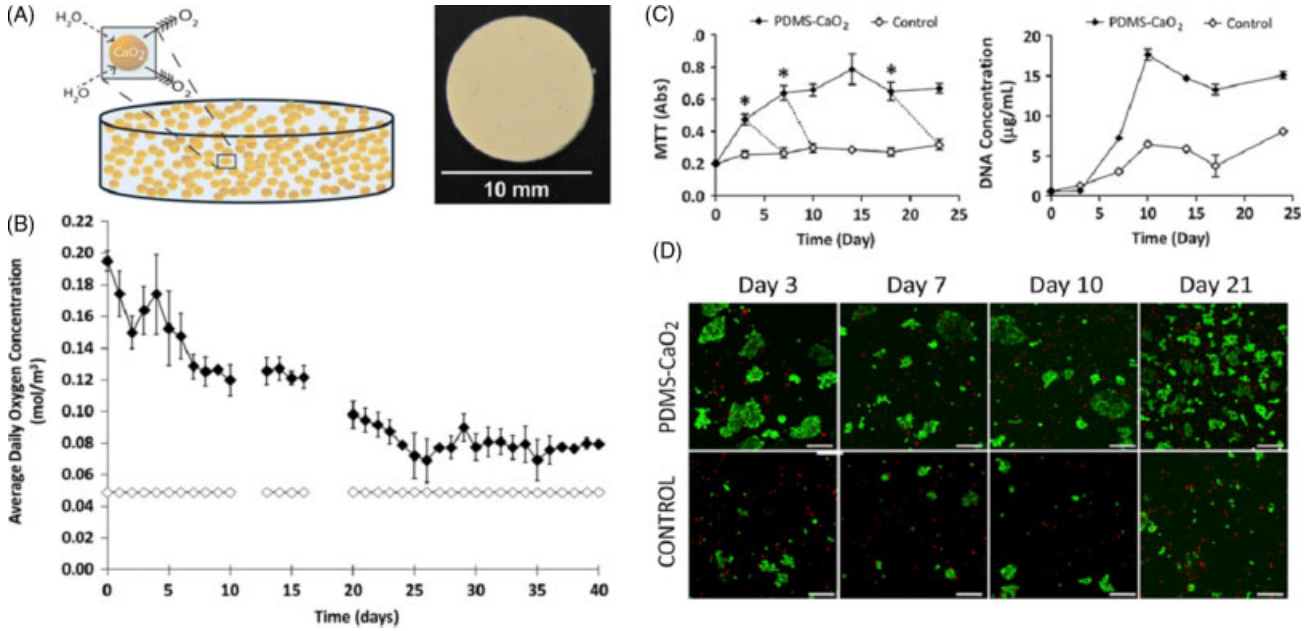

Figure 5. Oxygen-releasing agarose hydrogels. (A) CaO_2-encapulated PDMS disc. (B) Quantification of released oxygen over six weeks (filled diamonds, CaO_2-encapsulated PDMS discs; open diamonds, control PDMS discs without CaO_2). (C) Results for metabolic activity and DNA content assays for MIN6 beta cells encapsulated within oxygen-releasing agarose hydrogels over a three week time period. (D) Live/dead staining for MIN6 beta cells encapsulated within oxygen-releasing 3D agarose hydrogels at day 21. (Pedraza *et al*.[9] Copyright 2012 PNAS.)

The viability and proper function of cells, and conservation of cellular energy have important implications, especially during the early stages of engineered graft maturation and angiogenesis events. Oxygen-releasing tissue constructs are not only crucial for the fabrication of vascularized constructs, but also they are highly important for those cell types that demonstrate high metabolic activity and require high levels of oxygen for their survival and function. Therefore, consumption of oxygen within cell-loaded constructs significantly influences the cellular outcome. Thus, encapsulated cell type and cell density also affect the viability and growth processes. For example, beta-cells possess high metabolic activity and hence require elevated levels of oxygen for tissue formation.

In one study, Pedraza *et al*. generated CaO_2-encapsulated polydimethylsiloxane (PDMS) discs (Fig. 5(A)) and placed the oxygen-releasing cores inside beta-cell-laden agarose gels.[9] In that study, PDMS was chosen as the CaO_2-encapsulating polymer due to its hydrophobic properties that improve the efficiency of sustained release of oxygen by delaying the formation H_2O_2 upon contact with water. Steady release of oxygen from the CaO_2-encapsulated PDMS discs was monitored over an extended time period, for six weeks. In contrast, no change in oxygen levels was observed for the control PDMS discs (without CaO_2) in buffer solution (Fig. 5(B)). After confirmation of sustained release of oxygen, the cytocompatibility of the resulting biomaterial was tested by proliferation of MIN6 beta cells within oxygen-releasing hydrogels over three weeks, under both normoxic and hypoxic conditions. Cellular response was measured by total DNA content, metabolic activity (MTT; Fig. 5(C)), caspase activity and lactate dehydrogenase release assays. The results demonstrated that CaO_2-encapsulated PDMS augmented cell survival by preventing the formation of oxygen gradients throughout the hydrogel construct. As expected, scaffolds without CaO_2 showed significantly lower cell viability and proliferation. This approach could have important implications for transplantation of pancreatic cells to treat diabetes.

CONCLUSIONS AND FUTURE PERSPECTIVES

The inability to administer sufficient oxygen to thick artificial tissues and healing wounds has brought about a growing interest in the design and development of novel functional biomaterials. In this perspective, we have reviewed the major technologies that enable the incorporation of oxygen-releasing molecules into biomaterials for various tissue engineering applications. The elimination of the onset of hypoxia within engineered constructs from the time of implantation to the formation of functioning vasculatures is an exciting development towards translation into the clinic. It is expected that these strategies will open up new research avenues for numerous applications in regenerative engineering. With the recent advances, oxygen-releasing biopolymers are expected to significantly improve cell viability and tissue function in the future studies. These approaches are attractive for a wide range of areas in tissue engineering, such as wound healing, cardiac repair and beta-cell transplantation. It is anticipated that the demand for oxygen-releasing polymers will exponentially increase in the next few years due to the enormous need to fabricate off-the-shelf engineered products for regeneration/repair of various organs and tissues.

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