Enzyme-immobilized hydrogels to create hypoxia for *in vitro* cancer cell culture

Camron S. Dawes,¹ Heiko Konig,^{2,3} and Chien-Chi Lin^{1,3,*}

¹ Department of Biomedical Engineering, Indiana University-Purdue University Indianapolis, Indianapolis, IN, 46202, USA

² Department of Medicine, Division of Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN, 46202, USA

³ Indiana University Melvin & Bren Simon Cancer Center, Indianapolis, IN, 46202, USA

*To whom correspondence should be made:

Prof. Chien-Chi Lin
Associate Professor of Biomedical Engineering
Indiana University-Purdue University Indianapolis
723 W. Michigan St. SL220K
Indianapolis, IN 46202
Phone: (317)274-0760
Email: lincc@iupui.edu

Abstract

Hypoxia is a critical condition governing many aspects of cellular fate processes. The most common practice in hypoxic cell culture is to maintain cells in an incubator with controlled gas inlet (i.e., hypoxic chamber). Here, we describe the design and characterization of enzyme-immobilized hydrogels to create solution hypoxia under ambient conditions for *in vitro* cancer cell culture. Specifically, glucose oxidase (GOX) was acrylated and co-polymerized with poly(ethylene glycol)-diacrylate (PEGDA) through photopolymerization to form GOX-immobilized PEG-based hydrogels. We first evaluated the effect of soluble GOX on inducing solution hypoxia ($O_2 < 5\%$) and found that both unmodified and acrylated GOX could sustain hypoxia for at least 24 hours even under ambient air condition with constant oxygen diffusion from the air-liquid interface. However, soluble GOX gradually lost its ability to sustain hypoxia after 24 hours due to the loss of enzyme activity over time. On the other hand, GOX-immobilized hydrogels were able to create hypoxia within the hydrogel for at least 120 hours, potentially due to enhanced protein stabilization by enzyme 'PEGylation' and immobilization. As a proof-of-concept, this GOX-immobilized hydrogel system was used to create hypoxia for *in vitro* culture of Molm14 (acute myeloid leukemia (AML) cell line) and Huh7 (hepatocellular carcinoma (HCC) cell line). Cells cultured in the presence of GOX-immobilized hydrogels remained viable for at least 24 hours. The expression of hypoxia associated genes, including carbonic anhydrase 9 (CA9) and lysyl oxidase (LOX), were significantly upregulated in cells cultured with GOX-immobilized hydrogels. These results have demonstrated the potential of using enzyme-immobilized hydrogels to create hypoxic environment for *in vitro* cancer cell culture.

Keywords: Hypoxia; cancer; hydrogel; enzyme immobilization; glucose oxidase.

1. Introduction

Hypoxia, the lack of adequate oxygen (O_2) supply in cells and tissues, is a physiological condition of many healthy and diseased tissues in the body. For example, O_2 concentration is around 20% in the lungs; ~13% in the alveoli; ~5% in the circulation system and the bone marrow; and below 5% in multicellular tissues (De Miguel et al., 2015; Simon and Keith, 2008). Hypoxia is implicated in both normal physiological events and pathological conditions, including ischemia, tumors, and inflamed tissues. As such, O_2 concentration should be considered as a critical experimental condition when performing *in vitro* cell studies (Hockel and Vaupel, 2001; Liu and Simon, 2004; Semenza, 2000; Simon and Keith, 2008). Hypoxia stabilizes the expression of hypoxia inducible factors (HIFs) (Semenza, 2000), which are heterodimeric transcription factors that regulate many downstream genes and cell fate processes (Liu and Simon, 2004; Semenza, 2000), including proliferation, metabolism, apoptosis, stress response, angiogenesis, and migration. Hypoxia is also a key factor regulating tumor growth and drug resistance (De Miguel et al., 2015; Giaccia et al., 2003; Hockel and Vaupel, 2001; Liu and Simon, 2004).

The gold standard to induce hypoxia ($[O_2] < 5\%$) for *in vitro* cell culture is through using a cell culture chamber with controlled gas supplies (i.e., hypoxic chamber). However, the time needed to reach equilibrium of O_2 partial pressure between the chamber atmosphere and the culture medium could take several hours (Allen et al., 2001). Another challenge of using a hypoxic chamber is that O_2 diffusion from the air to the cell culture media occurs rapidly once the culture plates are removed from the hypoxic chamber. Unfortunately, studies have shown that even brief exposure of some cells to ambient air would cause drastic changes in certain hypoxia-related gene expression (Broxmeyer et al., 2015). For this reason, a glovebox is required if one wishes to maintain hypoxia throughout the experiment. The high front-end cost and dedicated space required for a hypoxic chamber system also limit its implementation to selected laboratories. In addition, it is challenging to perform real-time imaging or other

instrument-based live cell assays under hypoxia even with the use of a glovebox. Furthermore, one hypoxic chamber system can only provide one fixed O₂ tension for one experiment, which significantly retards the progress of scientific discovery related to varied O₂ tensions (e.g., hypoxia gradient, multiplex hypoxic drug testing, etc.).

Another method to induce hypoxia for cell culture is through introducing pre-equilibrated media with lower O₂ tension into the cell culture vessels, such as bioreactors or microfluidic devices. Bioreactors are the standard operation for scale-up production of cells or biological products but not ideal for mechanistic studies of hypoxia-induced cellular response. On the other hand, a microfluidic culture system permits real-time imaging of hypoxic cell culture and allows creation of complex hypoxia patterns within the confined microenvironment. For example, Peng *et al.* used chemical scavengers to reduce aqueous O₂ content within a patterned array of cells in wells of a microfluidic device with geometry matching that of a 96-well plate (Peng *et al.*, 2013). This method is beneficial in that multiple O₂ profiles can be developed rapidly through pre-equilibrated media in different wells of a single device. However, setting up microfluidic cell culture requires special instruments and project-specific microfluidic design. The applicability of such system in higher or enhanced-throughput analysis is also limited (e.g., drug screening and testing under various hypoxic conditions). It is also not an easy task to integrate microfluidic system with three-dimensional (3D) cell culture.

Hypoxic response in the cells can also be simulated using chemicals that upregulate or stabilize the expression of HIF1 α . Cobalt chloride (CoCl₂) or desferrioxamine are two examples of such chemicals (An *et al.*, 1998). Although this is a relatively simple strategy to mimic hypoxic response in the cells, the use of chemical only regulates cellular and molecular responses directly downstream of HIF. Furthermore, these chemicals could affect cell survival, metabolism, and morphology differently compared to real O₂ deprivation (Han *et al.*, 2006).

Recently, O₂-consuming enzymatic reactions are being developed as an alternative route to the aforementioned methods. The most notable example is the use of glucose oxidase

(GOX) and catalase (CAT) (Gibson et al., 1964; Kirkman and Gaetani, 2007). GOX oxidizes β -D-glucose while consuming O_2 to produce gluconic acid and hydrogen peroxide (H_2O_2). CAT is commonly added to reduce the cytotoxic H_2O_2 to one mole of water and a half mole of O_2 (**Figure 1A**). This system has been used to induce hypoxia in solutions and in microfluidic devices (Askoxylakis et al., 2011; Baumann et al., 2008; Huang et al., 2013; Li et al., 2016; Millonig et al., 2009; Mueller et al., 2009; Rajan et al., 2013; Sobotta et al., 2013; Zitta et al., 2012). The use of GOX/CAT is beneficial in that the system provides a rapid onset of hypoxia (usually within a few minutes) (Askoxylakis et al., 2011; Baumann et al., 2008; Huang et al., 2013; Li et al., 2016; Millonig et al., 2009; Mueller et al., 2009; Rajan et al., 2013; Sobotta et al., 2013; Zitta et al., 2012). One drawback to any GOX system, however, is the production of hydrogen peroxide, a reactive oxygen species (ROS) (Fruehauf and Meyskens, 2007) whose accumulation would not only cause undesired cellular response but also inactivate both GOX and CAT (Hielscher and Gerecht, 2015; Pal et al., 2000; Trachootham et al., 2009; Tse and Gough, 1987). Thus far, the applications of GOX/CAT system have been focused on glucose sensing and pH-induced responses (Wu et al., 2011). For example, Choi *et al.* prepared GOX-immobilized poly(ethylene glycol) (PEG) hydrogels and studied the effect of gel compositions on immobilized enzyme activity. Although the production of H_2O_2 was quantified to evaluate kinetics of the immobilized enzyme kinetics, O_2 contents were not monitored (Choi et al., 2008). Some recent work has started to explore the ability of GOX/CAT reactions to induce hypoxia for *in vitro* cell culture (Askoxylakis et al., 2011; Baumann et al., 2008; Huang et al., 2013; Li et al., 2016; Millonig et al., 2009; Mueller et al., 2009; Rajan et al., 2013; Sobotta et al., 2013). The GOX/CAT system has also been adapted to 3D printed inserts (Li et al., 2016) where GOX and CAT were coated on printed disks and the degrees of solution hypoxia were controlled by the distance between the enzyme-immobilized disks and the solution in the culture plate. In that design, hypoxia conditions (between 0 and ~12% O_2) were maintained for up to 5 hours and the system was used to induce hypoxic response in peritoneal macrophages.

Other enzymes (e.g., laccase) have also been used to create hypoxia (Blatchley et al., 2015; Park et al., 2014; Park and Gerecht, 2014). In the laccase system, a fixed amount of substrate (i.e., ferulic acid, FA) was immobilized to a polymer backbone. The FA-immobilized polymer was then crosslinked by laccase-mediated enzymatic reaction, which also consumes O₂. Recently, Lewis *et al.* extended the timespan of laccase-induced hypoxia by limiting the diffusion of O₂ into the FA-crosslinked hydrogel. Together with the enzymatic O₂ depletion, the system was successfully used to study the impact of hypoxia on sarcoma cell invasion and migration (Lewis et al., 2016).

Here, we present an immobilized enzyme strategy for inducing hypoxia within and surrounding the PEG-based hydrogel for *in vitro* cancer cell culture. Immobilization of oxygen-consuming GOX within covalently crosslinked hydrogels provide an easy method to control solution oxygen tension without using external devices. Furthermore, GOX-immobilized hydrogels can be readily added to or removed from cell culture without disturbing cells. The crosslinked PEG hydrogel network also provides opportunities for immobilizing multiple proteins/enzymes or other functional molecules for other biomedical applications. In this contribution, we systematically studied the ability of GOX, PEG-acrylate modified GOX (i.e., GOX_{PEGA}), and GOX-immobilized hydrogels to induce solution hypoxia. Furthermore, we utilized GOX-immobilized hydrogels to induce hypoxia for *in vitro* culture of anchorage-independent acute myeloid leukemia cells Molm14 and anchorage-dependent hepatocellular carcinoma cells Huh7. We evaluated the effects of GOX-immobilized hydrogels on cancer cell fate, including viability, proliferation, and hypoxia-associated gene expression.

2. Materials & methods

Linear PEG (Mn = 2 kDa) was purchased from Sigma-Aldrich. Glucose oxidase (0243-500KU) and catalase (LS001847) were purchased from Amresco and Worthington Biochemical, respectfully. Acrylate-PEG-succinimidyl valerate (Acryl-PEG-SVA, MW 3,400 Da) was obtained

from Laysan Bio Inc. Zeba Spin Desalting Columns (7K MWCO), 2,4,6-trinitrobenzene sulfonic acid (TNBSA), and β -D-glucose were purchased from Thermo Scientific. Penicillin-streptomycin, antibiotic-antimycotics, fetal bovine serum (FBS), Roswell Park Memorial Institute media (RPMI), and Dulbecco's modified Eagle's medium (DMEM) were acquired from Life Technologies. HEPES and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Lonza. Membrane culture plate inserts (PIXP-012-50) were purchased from EMD Millipore. Tryphan blue and AlamarBlue® reagents were purchased from Mediatech and Fisher Scientific, respectively.

2.1 Macromer synthesis and characterization

PEG-diacrylate (PEGDA) was synthesized according to an established protocol (Hao and Lin, 2014) and characterized with $^1\text{H-NMR}$ (Bruker 500). The degree of PEGDA functionalization was around 89% (**Figure S1**). Photoinitiator lithium aryl phosphonate (LAP) was synthesized as described elsewhere (Fairbanks et al., 2009).

To facilitate enzyme immobilization within hydrogels, glucose oxidase was acrylated using Acryl-PEG-SVA (Choi et al., 2008). Briefly, the enzyme was first dissolved at 20 mg/mL in PBS supplemented with 2 mM EDTA (pH 8.5) and 50 mM sodium carbonate. Acryl-PEG-SVA was added at 200x molar excess to enzyme concentration and the reaction was allowed to proceed at room temperature for 2 hours with stirring. During the reaction, primary amines on the surface of the enzyme reacted with SVA groups to afford PEG-acrylate (PEGA)-modified GOX (GOX_{PEGA}). Unreacted macromers were removed using size exclusion chromatography columns (Zeba Spin Desalting column). Un-modified GOX at the same concentration was also passed through the columns and used as controls to account for any loss/entrapment of enzyme within the columns. Following synthesis, both GOX and GOX_{PEGA} were assayed using TNBSA assay to determine the degree of PEGA functionalization. For each assay, enzyme samples were diluted to 30-35 $\mu\text{g/mL}$. A series of lysine hydrochloride solutions (0-10 $\mu\text{g/mL}$,

200 μL /well) were used as standards. 100 μL of 0.01% TNBSA reagent was added into wells of a 96-well plate, which was sealed and incubated at 37°C for 2 hours, followed by cooling for 5 minutes. Absorbance at 335 nm was measured using a microplate reader (SynergyHT BioTek). The degree of PEGA functionalization on GOX was determined as the concentration of remaining amine groups on GOX_{PEGA} over that of the un-modified GOX.

2.2 Characterization of enzymatic activity of GOX_{PEGA}

To examine the enzyme activity, O₂ consumption in the presence of the enzyme and glucose was quantified. The changes in O₂ content over time in the presence of GOX or GOX_{PEGA} ($V_o = \Delta[\text{O}_2] / \Delta\text{Time}$) was defined as the reaction velocity. Enzyme was dissolved PBS (pH 7.4) at 0.13 μM in a 2 mL microtube with constant stirring at 25°C. The oxygen consumption reactions were carried out under ambient air with constant oxygen diffusion from the air to mimic actual cell culture condition. Stock β -D-glucose solution was injected at the start of every measurement to give starting concentrations of 0.30 to 25 mM [S]_i. Dissolved O₂ concentration was monitored for 3 minutes using an O₂ probe and meter (Microx4, PreSens). O₂ contents were plotted as a function of time and the initial linear portion of the curve was used for V_o calculation (change in substrate concentration over time). Non-linear regression analysis and curve fitting was applied to paired V_o and [S]_i using the equation $V_o = V_{\text{max}} \cdot [\text{S}] / (K_m + [\text{S}])$. In the equation, V_{max} is the theoretical maximum enzyme reaction velocity and K_m is the Michaelis-Menten constant, the equilibrium dissociation constant (i.e., affinity) for the enzyme and the substrate.

2.3 Synthesis and characterization of enzyme-immobilized hydrogels

All macromer solutions were sterilized by passing through 0.22 μm syringe filters. PEGDA hydrogels (15 wt%) were polymerized aseptically through radical mediated photopolymerization in the absence or presence of GOX_{PEGA} monomer (6 mg/mL), and LAP (1 mM) as the photoinitiator. 60 μL gels were injected between two glass slides separated by

Teflon spacers (2 mm) and gelation was initiated with a UV lamp (365 nm, 5 mW/cm², 2 min exposure). Following photopolymerization, hydrogels (~ 3.1 mm dia. x 2 mm thickness) were incubated in DPBS for 24 hours at 37°C.

O₂ concentration in solution was measured with a dipping-type O₂ sensor (Microx4, PreSens). For solution based measurements, the probe was extended to ~2 mm above the bottom of the 24 well plate or 1 mm above the gel (~2 mm from the liquid-air interface). To measure the H₂O₂ produced during the reactions, 10 µL aliquots of the solutions were collected and quantified with a Quantichrom Peroxide Assay Kit following the manufacturer's protocol (BioAssay Systems).

2.4 Cell culture and viability assays

A suspension cell type, human acute myeloid leukemia (AML) cells Molm14, was purchased from Leibniz Institute, German Collection of Microorganisms and Cell Cultures. Cells were maintained in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin 25 mM HEPES, and 25 mM β-D-Glucose. 400,000 cells/mL of Molm14 cells were seeded per well in non-treated 24 well plates. GOX_{PEGA} gels (15 wt% PEGDA, 6 mg/mL GOX_{PEGA}, 60 µL per gel) were added to half of the wells (one gel per well) containing 0.54 mg/mL catalase. Remaining wells were placed with gels without immobilized enzyme. *In vitro* O₂ concentration was measured ~1 mm above the hydrogel with a dipping-type O₂ sensor (PreSens). Adherent cell type human hepatocellular carcinoma cells (Huh7) were grown in high glucose DMEM supplemented with 10% FBS, 1% antibiotic antimycotics, and 25 mM HEPES. Cells were seeded on treated 24 well plates with 1 mL per well of cell suspension (60,000 cells/mL) and allowed to grow/spread for 48 hours prior to the onset of the experiments, at which time (labeled as 0 hours) culture media was refreshed in all wells. At the onset of the experiment, membrane inserts containing GOX_{PEGA} gels were placed in the wells and the

medium was supplemented with 0.54 mg/mL CAT. Half of the wells only had media refreshed and were used as control groups for the experiment (no enzyme added).

Molm14 cell viability and density were characterized by tryphan blue staining and counting with a hemocytometer. AlamarBlue® reagent (10x dilution in media) was used for assaying metabolic activity of Huh7 cells. After a 90 min incubation, 200 μ L from each well was transferred to a clear 96-well microplate and read for fluorescence (excitation/emission: 560/590 nm).

2.5 RNA isolation and real time PCR

RNA isolation was carried out using NucleoSpin RNA II kit (Clontech). Briefly, 600 μ L of lysis buffer was added to each well containing cells. Cell lysates were snap frozen and stored in -80°C until assay. After thawing the lysates, 600 μ L of 70% RNase free ethanol was added, pipetted vigorously, and then run through NucleoSpin RNA columns. After desalting/purification steps, RNA was eluted with DNase/RNase-free H_2O and quantified by spectroscopy (NanoDrop 2000, Thermo Scientific). Isolated RNA was stored at -80°C .

Complementary DNA was generated from the isolated total RNA by using PrimeScript RT reagent kit (Clontech, TaKaRa). Gene expression was analyzed by real time quantitative PCR using SYBR Premix Ex Taq II Kit (Clontech, TaKaRa). The kit components, cDNA, and primers were mixed in a PCR plate and analyzed on a 7500 Fast Real-Time PCR machine (Applied Biosystems). Thermocycling parameters were one cycle at 95°C for 30s, followed by 95°C for 3s, 60°C for 30s, and repeat for 45 cycles. Gene expression results were analyzed using $2^{-\Delta\Delta\text{CT}}$ methodology. For each experimental condition, cycle count was first standardized to ribosomal 18S housekeeping gene (ΔCT level) and then normalized with respect to the media control group for that specific time point ($\Delta\Delta\text{CT}$ level; media control values were set as one-fold). **Table S1** lists all primer sequences used for real-time PCR.

2.6 Statistics

GraphPad Prism 5 was used for all curve fitting and statistical analyses. Significance comparison between experimental groups was performed using Two-Way ANOVA with Bonferroni post testing. Michaelis-Menten parameters for GOX and GOX_{PEGA} were generated with the non-linear regression suite in GraphPad Prism 5 software. All experiments were conducted a minimum of three times with data presentation as the mean $\pm$ standard error of the mean (SEM). One, two, or three asterisks represent $p < 0.05$, 0.01 , or 0.001 , respectively.

3. Results

3.1 Enzyme-induced hypoxia

GOX/CAT systems have been used to induce pH changes in aqueous environment due to the production of gluconic acid (**Figure 1A**). To gain insights into the capability of this enzymatic system on inducing hypoxia in solution, we quantified the reduction of O₂ in aqueous buffers supplemented with different concentrations of GOX in the absence (**Figure 1B**) or presence (**Figure 1C**) of CAT. The O₂ tensions of the buffers were monitored using an O₂ sensor and recorded as a function of time. As expected, higher concentrations of GOX were able to deplete O₂ faster. As shown in **Figure 1B**, O₂ tension was reduced within 5 minutes to ~6% or to ~2.5% when 2.25 $\mu\text{g/mL}$ or 4.5 $\mu\text{g/mL}$ of GOX was added, respectively. With the inclusion of 9.8 $\mu\text{g/mL}$ of CAT in the solution, the rate of O₂ consumption was slowed down to ~9% and to 3.2% within 5 minutes (**Figure 1C**). The decrease in O₂ consumption is not surprising as the addition of CAT produces one-half mole of O₂ per mole of H₂O₂ consumed.

In order to fabricate enzyme-immobilized hydrogels capable of inducing hypoxia, the primary amine groups on GOX were functionalized with 200-fold molar excess of Acryl-PEG-SVA (**Figure 2A**) (Choi et al., 2008). TNBSA assay results showed an average of $93 \pm 1.7\%$ (Mean $\pm$ SEM, $N = 5$) of the primary amines on enzyme surface were functionalized with Acryl-PEG. The acrylate moieties on the surface of Acryl-PEG-GOX (i.e., GOX_{PEGA}) permit its homopolymerization with PEGDA to afford enzyme-immobilized hydrogels. The modification,

however, could affect the ability of GOX_{PEGA} to consume O₂. As shown in **Figure 2B**, while unmodified GOX caused rapid O₂ reduction (from ~20% to ~3.2% in 5 minutes) in solution, the ability of GOX_{PEGA} to consume O₂ was slightly hindered after Acryl-PEG-SVA modification (from ~20% to ~5.9% within 5 minutes). To quantify the impact of polymer modification on its enzyme activity, reaction velocities of GOX and GOX_{PEGA} were measured and compared in **Figure 2C**. Michaelis-Menten enzyme kinetic parameters were listed in **Table 1**. Maximum reaction velocity, $V_{\max}$, was reduced for GOX_{PEGA} to 0.664 mM·min⁻¹, or approximately 75% of that for GOX (0.880 mM·min⁻¹). Additionally, K_m , an estimate of the dissociation constant for enzyme and substrate, was also decreased for GOX_{PEGA} at 1.1173 mM versus GOX at 4.380 mM.

To evaluate the ability of the enzyme system to maintain hypoxia, O₂ content measurements were carried out for 72 hours. **Figure 3** shows long term solution hypoxia induced by GOX or GOX_{PEGA} in the absence (**Figure 3A**) and presence (**Figure 3B**) of CAT. Between GOX and GOX_{PEGA} groups, O₂ content was very similar for all time points. Within the first 24 hours, O₂ was maintained below 5% but gradually increased to ~13% by 72 hours (**Figure 3A**). The addition of CAT did not affect O₂ content, which was below 5% in the first 24 hours for both GOX and GOX_{PEGA}. The O₂ content in both conditions rose to ~16% and ~18% at 50 and 72 hours, respectively.

3.2 GOX_{PEGA}-immobilized hydrogels for inducing hypoxia

GOX_{PEGA} was covalently immobilized within PEGDA hydrogels to provide a simple method for inducing solution hypoxia. The ability of the immobilized enzyme to reduce O₂ in the surrounding solution and within the gel was measured with a needle type optical probe as shown in **Figure 4A**. With the needle type O₂ probe, it was possible to measure O₂ content outside (left panel) or inside (right panel) the GOX_{PEGA} immobilized hydrogels (**Figure 4B**). Control experiments using hydrogels without enzyme immobilization (i.e., (-) GOX_{PEGA}) showed that O₂ content remained close to normoxia (17-20% O₂, **Figure S2**). Furthermore, there was no

significant difference between O₂ content within or outside of the enzyme-free hydrogels. With the use of GOX_{PEGA} immobilized PEG hydrogels, however, there was a rapid drop in the ‘exterior’ (i.e., outside of the GOX_{PEGA} immobilized hydrogel) O₂ tension within one hour, a level similar to that with soluble enzyme (**Figure 3A**). O₂ tension was roughly at ~8% O₂ for 48 hours in solution with the GOX_{PEGA} hydrogels. Conversely the O₂ tension within the GOX_{PEGA} hydrogel quickly reached and maintained near anoxia (~0% O₂) for 48 hours. The O₂ tension at the gel exterior had increased to ~15% by 120 hours, while that in the gel interior was still below 2%.

3.3 Combined GOX_{PEGA}-immobilized hydrogels and soluble CAT for inducing hypoxia

Next, GOX_{PEGA}-immobilized hydrogels were placed in buffer solution containing catalase and the solution O₂ content was measured (**Figure 5A**). After placing the GOX_{PEGA} gel, hypoxia was induced and sustained for at least 6 hours. By 24 hours, the O₂ content in solution without CAT returned to ~14%, whereas it remained ~6% in the presence of CAT. By 48 hours, the O₂ content returned to ~14% and ~16% for solution with or without CAT, respectively. Increasing percent O₂ over time would indicate that some degree of enzyme activity was lost. **Figure 5B** shows that, in the presence of CAT, H₂O₂ concentrations were 2 mM and 5 mM at 24 and 48 hours, respectively. In the absence of CAT, however, H₂O₂ concentration increased to ~9 mM at both time points. To ensure that glucose was not exhausted during the GOX reaction, we supplemented the solution with additional D-glucose at later time points. A 50 µL shot of β-D-glucose (at 50 mM) was added to the solution (1000 µL) containing GOX_{PEGA} gel 5 minutes before O₂ detection (at 24 and 48 hours). As shown in **Figure 5C and 5D**, bolus addition of glucose did not significantly affect the O₂ content or the H₂O₂ concentration in solution.

To test whether replacing a new GOX_{PEGA} gel would prolong hypoxia in the solution, we removed the old gel after 24 hours and placed a freshly prepared GOX_{PEGA} gel (identical formulation). We also added freshly prepared CAT solution. **Figure S3** shows that the O₂ content was maintained at ~5% at 30 hours (i.e., 6 hours post gel replacement), whereas the O₂

content was ~9% in the control group (i.e., without replacing GOX_{PEGA} gels). However, the O₂ content in both conditions increased to above 14% at 48 hours.

3.4 Cytocompatibility of enzyme-immobilized hydrogels

Figure 6 shows the cytocompatibility of enzyme-free (i.e., PEGDA only) and GOX-immobilized hydrogels. Molm14 cell viability was maintained above 95% over the course of 48 hours in the presence of an enzyme-free PEGDA hydrogel (**Figure 6A**). These cells were also proliferating over time, as indicated by steady increase in cell density (**Figure 6B**). When a GOX_{PEGA} gel was placed together with Molm14 cells (with media-supplemented CAT), cell viability in the initial 24 hours was comparable to that in the media-only control (around 90%, **Figure 6C**). However, after 48 hours of *in vitro* culture, Molm14 cell viability declined sharply to ~55%. In addition to the decreased cell viability after 48 hours, a similar trend can be seen with cell density over time (**Figure 6D**). Specifically, there was no significant difference in cell density between the control and experimental group at 6 hours (i.e., $\sim 3.6 \times 10^5$ cells/mL). By 48 hours the Molm14 cell density in the media-only control group had increased to $\sim 5.5 \times 10^5$ cells/mL, whereas the Molm14 cell density in the GOX-immobilized hydrogel group decreased significantly to $\sim 2.2 \times 10^5$ cells/mL.

3.5 Enzyme-induced hypoxia in the presence of cells

The main focus of this study was to develop an enabling material technology for facilitating a wide variety of *in vitro* cancer cell culture. We choose anchorage-independent acute myeloid leukemia (AML) cell line Molm14 and anchorage-dependent hepatocellular carcinoma (HCC) cell line Huh7 to test the utility of the hypoxia-inducing hydrogels. The survival and progression of these cells, just like many other cancer cell types, were significantly affected by oxygen tension. GOX_{PEGA} immobilized hydrogels were prepared and added to anchorage-independent Molm14 cells cultured directly. As shown in **Figure 7A**, solution hypoxia was rapidly induced and maintained below 5% O₂ from 6 to 24 hours. By 48 hours, however, O₂

concentration had risen to near normoxia (17-20% O₂), a result consistent with the cell-free measurements shown in **Figure 5A and 5C**. For anchorage-dependent cell Huh7, GOX_{PEGA}-immobilized hydrogel was placed in a standard transwell device and co-cultured with the cells adhered to the surface of a multi-well plate. The purpose of using a transwell device was to prevent direct contact of the gel with the cells, which could mechanically disrupt cell attachment. **Figure 7C** shows that O₂ profile development was similar to that for Molm14 cells. Low O₂ concentration was reached quickly and maintained up to 24 hours. By 48 hours, the O₂ content had returned to almost normoxia. As expected, the addition of CoCl₂ in cell culture media did not change O₂ tension (**Figure 7C**).

3.6 Effect of enzyme-induced hypoxia on hypoxic gene expression

In addition to cell viability, the expression of hypoxia associated gene carbonic anhydrase 9 (CA9) in Molm14 cells was evaluated at 6 and 24 hours of culture in the presence of a GOX-immobilized hydrogel. As shown in **Figure 7B**, enzyme-induced hypoxia increased the expression of CA9 significantly compared with control groups (~3-fold and ~10-fold higher at 6 and 24 hours of culture, respectively). For Huh7 cells, the expression of carbonic anhydrase 9 (CA9) and lysyl oxidase (LOX) was examined after the cells were exposed to the enzyme-immobilized hydrogel (Note: no detectable LOX expression was found in Molm14 cells). In selected groups, CoCl₂ was added as another control for chemically stimulated hypoxic response. As shown in **Figure 7D**, CoCl₂ failed to upregulate CA9 expression in the first 24 hours. After the same period of time in culture, the use of GOX_{PEGA} gels + CAT led to a ~20-fold increase in CA9 expression in Huh7 cells. After 48 hours, the addition of CoCl₂ caused ~15-fold upregulation in CA9 mRNA expression, which was much lower than that induced by the enzyme-immobilized hydrogel group (~80-fold higher). In Huh7 cells, LOX mRNA expression was upregulated only in cells co-cultured with a GOX_{PEGA} gel (~2.5 fold, **Figure S4**). The addition of CoCl₂ did not increase the expression of LOX in Huh7 cells.

4. Discussion

This work aims to develop an enabling material strategy to create solution hypoxia for *in vitro* cancer cell culture without using complex devices/instrument. GOX-immobilized hydrogel was used as a proof-of-concept to demonstrate the ability of enzyme-immobilized gel to induce sustained hypoxia. We established the concentration of GOX (4.5 μg in 1 mL buffer) required to induce hypoxia (<5%) within 5 minutes (**Figure 1B**). As expected, the addition of CAT partially replenished O_2 , leading to slight decrease in reaction velocity (**Figure 1C**). Chemical modification is necessary for the immobilization of GOX into hydrogels but significant modification might result in the loss of enzymatic activity, especially when the degree of enzyme modification that we have achieved was more than 90% of the available amine groups on enzyme surface. Analyses of enzyme reaction kinetics revealed that chemical modification (i.e., PEG-acrylation) of GOX only caused slight reduction of its O_2 consumption ability (**Figure 2 & Table 1**). The kinetic parameters of GOX obtained from this study were relatively low compared to values reported in the literature. (Gibson et al., 1964). This could be attributed to the differences in experimental conditions and the methods for which enzyme activities were measured. Conventionally, the activity of GOX is assayed by monitoring β -D-glucose concentration, whereas in the current study we detected O_2 concentration. Although the oxygen contributed from atmospheric air or from reduced H_2O_2 (by CAT or natural reduction with a half-life of ~10 minutes in cell culture environment, Mueller et al., 2009) would complicate the analysis results, the current detection method was necessary as the main purpose of this project was to investigate whether enzyme-immobilized hydrogels could induce solution hypoxia for *in vitro* cell culture under ambient conditions (i.e., with O_2 diffusion from the air). The slight reduction of activity in modified enzyme was likely caused by changes in secondary and tertiary protein structure following significant GOX surface modification (Pandey et al., 2007). Nonetheless, both unmodified GOX and GOX_{PEGA} were able to induce solution hypoxia (O_2 < 5%) within a few minutes (**Figure 2B**) and solution hypoxia was sustained for up to 24 hours

(**Figures 3**). Additionally, no statistically significant difference was observed between GOX and GOX_{PEGA} activity within the first 24 hours (**Figure 3**). Our results were consistent to that reported by Kang *et al.* where methacryloyl-modified GOX exhibited similar enzymatic activity in solution compared with unmodified GOX (Kang and Bae, 2003). We also found that GOX_{PEGA} co-polymerized in PEGDA hydrogels was able to induce hypoxia rapidly after dropping the gel in glucose-containing buffer solution. However, GOX-immobilized hydrogel could only sustain solution hypoxia for 24 hours. The restoration of O₂ tension in the solution was not due to the lost of enzyme activity as O₂ tension at the interior of the GOX-immobilized hydrogel was maintained below 2% for up to 5 days (**Figure 4B**). It was likely that constant oxygen diffusion from the liquid-air interface supersedes oxygen consuming enzymatic reaction in the hydrogel. This hypothesis was supported by the fact that replacing the old gel with a freshly prepared GOX-immobilized hydrogel only prolonged hypoxia for a few hours (**Figure S3**). Another possible reason was that some enzymes were deactivated by the accumulation of acid by-product gluconic acid and H₂O₂. To test whether H₂O₂ deactivated the enzyme, CAT was introduced in the solution to consume H₂O₂ (**Figure 5**). The increase of O₂ content in the solution (**Figure 5A**) coincided with the accumulation of H₂O₂ levels (**Figure 5B**), supporting the likelihood of enzyme deactivation by elevated H₂O₂ concentration. This phenomenon has been reported in membrane-bound GOX and CAT system where both enzyme activities were reduced with increased H₂O₂ concentrations over time (Blandino et al., 2002; Tse and Gough, 1987). It was not likely that the enzymatic reactions depleted most of the substrate (i.e., β -D-glucose) after 24 hours, as the addition of extra β -D-glucose periodically before O₂ tension measurement did not cause further O₂ consumption. Whether there was additional glucose addition or not, no statistically significant difference was found in O₂ (**Figure 5C**) or H₂O₂ concentration (**Figure 5D**).

To demonstrate the utility of this immobilized enzyme system on *in vitro* cell culture, we characterized cell viability, proliferation, and the expression of hypoxia-regulated genes (i.e.,

CA9 or LOX) in cells cultured in the presence of GOX-immobilized hydrogels. Both CA9 and LOX are downstream targets of HIF1 α and are well-established genes associated with cancer metastasis and metabolism in hypoxic environments (Erler et al., 2006; Wykoff et al., 2000). Detecting the expression of these two genes allowed us to evaluate the utility of our hypoxia-inducing hydrogels. Results show that the expression of CA9 and LOX was significantly higher in Huh7 cells cultured with GOX-immobilized hydrogels (**Figures 7D & S4**). Molm14 cells also had increased CA9 expression (**Figure 7B**). However, no expression of LOX was detected (control group included); there is also a lack of literature for LOX expression with Molm14 cells or the sister Molm13 cell line. Nonetheless, similar data for both CA9 and LOX expression was reported by Askoxylakis *et al.* where GOX and CAT were dissolved in culture media to generate hypoxia for culturing head and neck squamous carcinoma cells (Askoxylakis et al., 2011). Although GOX-immobilized hydrogel was effective in inducing solution hypoxia under ambient conditions, these gels appeared to hinder cell viability and proliferation after the cells were exposed to the hydrogel system for longer than 24 hours. This was most likely attributed to the accumulation of cytotoxic byproduct, namely gluconic acid and H₂O₂. This is not a unique problem to our system as other GOX/CAT-induced hypoxia culture systems (e.g., soluble or membrane-bound enzyme) also exhibited decreased cytocompatibility after 24 hours of culture (Askoxylakis et al., 2011; Huang et al., 2013; Zitta et al., 2012). One potential solution for this drawback is to replace the GOX-immobilized hydrogel and the CAT supplemented media frequently. Providing fresh media and hydrogel allows hypoxia to quickly be reestablished and maintained for periods lasting for at least 24 hours. Furthermore, media acidification by gluconic acid could be reduced by supplementing cell culture media with HEPES, which provides strong buffering effect without altering normal cell physiology.

Lastly, other O₂-consuming enzymes (e.g., laccase, bilirubin oxidase, polyphenol oxidase, catechol oxidase, etc.) could be immobilized in hydrogels for inducing solution hypoxia. One benefit of these enzymes is that they do not produce H₂O₂, which would provide for longer

hypoxia without enzymatic inactivation or adverse effects of reactive oxygen species on cells. Additionally, different reaction speeds corresponding to specific enzymes can be used to create fast or slow hypoxia induction.

5. Conclusion

In summary, we have developed enzyme-immobilized hydrogels to rapidly induce hypoxia for *in vitro* cell culture. Even when the solution was kept under ambient air, the GOX-immobilized hydrogel was able to establish and sustain *in vitro* hypoxic conditions (<5% O₂) for 6 to 24 hours, a time frame sufficient for many biological characterizations and assays. Although PEGDA hydrogel retains and stabilizes GOX in the gel for at least five days, a continuous supply of O₂ from the air eventually overcomes the enzymatic O₂ reduction. Furthermore, the accumulation of reaction by-products and glucose depletion might limit the utility of the current system to applications within 24 hours. Regardless, enzyme-immobilized hydrogels provide a simple, flexible, and more efficient option to induce realistic hypoxia compared with the use of chemical compounds. Future work will focus on prolonging the timespan of hypoxia through system optimization or via the use of other oxygen consuming enzymes.

Acknowledgments

This work was supported by the National Science Foundation (CAREER Award, DMR #1452390, to CL), Leukemia & Lymphoma Society (New Idea Award, to HK), and the Department of Biomedical Engineering at IUPUI (Graduate Research Fellowship, to CSD). The authors thank Dr. Tsai-Yu Lin for his assistance in real-time PCR.

References

- Allen, C.B., Schneider, B.K., White, C.W., (2001) Limitations to oxygen diffusion and equilibration in in vitro cell exposure systems in hyperoxia and hypoxia. *American journal of physiology. Lung cellular and molecular physiology* 281, L1021-1027.
- An, W.G., Kanekal, M., Simon, M.C., Maltepe, E., Blagosklonny, M.V., Neckers, L.M., (1998) Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature* 392, 405-408.
- Askoxylakis, V., Millonig, G., Wirkner, U., Schwager, C., Rana, S., Altmann, A., Haberkorn, U., Debus, J., Mueller, S., Huber, P.E., (2011) Investigation of tumor hypoxia using a two-enzyme system for in vitro generation of oxygen deficiency. *Radiation oncology* 6, 35.
- Baumann, R.P., Penketh, P.G., Seow, H.A., Shyam, K., Sartorelli, A.C., (2008) Generation of oxygen deficiency in cell culture using a two-enzyme system to evaluate agents targeting hypoxic tumor cells. *Radiation research* 170, 651-660.
- Blandino, A., Macías, M., Cantero, D., (2002) Modelling and simulation of a bienzymatic reaction system co-immobilised within hydrogel-membrane liquid-core capsules. *Enzyme and microbial technology* 31, 556-565.
- Blatchley, M., Park, K.M., Gerecht, S., (2015) Designer Hydrogels for Precision Control of Oxygen Tension and Mechanical Properties. *Journal of materials chemistry. B, Materials for biology and medicine* 3, 7939-7949.
- Broxmeyer, H.E., O'Leary, H.A., Huang, X., Mantel, C., (2015) The importance of hypoxia and extra physiologic oxygen shock/stress for collection and processing of stem and progenitor cells to understand true physiology/pathology of these cells ex vivo. *Current opinion in hematology* 22, 273-278.
- Choi, D., Lee, W., Park, J., Koh, W., (2008) Preparation of poly(ethylene glycol) hydrogels with different network structures for the application of enzyme immobilization. *Bio-medical materials and engineering* 18, 345-356.

- De Miguel, M.P., Alcaina, Y., de la Maza, D.S., Lopez-Iglesias, P., (2015) Cell metabolism under microenvironmental low oxygen tension levels in stemness, proliferation and pluripotency. *Current molecular medicine* 15, 343-359.
- Erler, J.T., Bennewith, K.L., Nicolau, M., Dornhofer, N., Kong, C., Le, Q.-T., Chi, J.-T.A., Jeffrey, S.S., Giaccia, A.J., (2006) Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* 440, 1222-1226.
- Fairbanks, B.D., Schwartz, M.P., Bowman, C.N., Anseth, K.S., (2009) Photoinitiated polymerization of PEG-diacrylate with lithium phenyl-2,4,6-trimethylbenzoylphosphinate: polymerization rate and cytocompatibility. *Biomaterials* 30, 6702-6707.
- Fruehauf, J.P., Meyskens, F.L., Jr., (2007) Reactive oxygen species: a breath of life or death? *Clinical cancer research : an official journal of the American Association for Cancer Research* 13, 789-794.
- Giaccia, A., Siim, B.G., Johnson, R.S., (2003) HIF-1 as a target for drug development. *Nature reviews. Drug discovery* 2, 803-811.
- Gibson, Q.H., Swoboda, B.E., Massey, V., (1964) Kinetics and Mechanism of Action of Glucose Oxidase. *The Journal of biological chemistry* 239, 3927-3934.
- Han, Y.H., Xia, L., Song, L.P., Zheng, Y., Chen, W.L., Zhang, L., Huang, Y., Chen, G.Q., Wang, L.S., (2006) Comparative proteomic analysis of hypoxia-treated and untreated human leukemic U937 cells. *Proteomics* 6, 3262-3274.
- Hao, Y., Lin, C.C., (2014) Degradable thiol-acrylate hydrogels as tunable matrices for three-dimensional hepatic culture. *Journal of biomedical materials research. Part A* 102, 3813-3827.
- Hielscher, A., Gerecht, S., (2015) Hypoxia and free radicals: role in tumor progression and the use of engineering-based platforms to address these relationships. *Free radical biology & medicine* 79, 281-291.

- Hockel, M., Vaupel, P., (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *Journal of the National Cancer Institute* 93, 266-276.
- Huang, Y., Zitta, K., Bein, B., Steinfath, M., Albrecht, M., (2013) An insert-based enzymatic cell culture system to rapidly and reversibly induce hypoxia: investigations of hypoxia-induced cell damage, protein expression and phosphorylation in neuronal IMR-32 cells. *Disease models & mechanisms* 6, 1507-1514.
- Kang, S.I., Bae, Y.H., (2003) A sulfonamide based glucose-responsive hydrogel with covalently immobilized glucose oxidase and catalase. *Journal of Controlled Release* 86, 115-121.
- Kirkman, H.N., Gaetani, G.F., (2007) Mammalian catalase: a venerable enzyme with new mysteries. *Trends in biochemical sciences* 32, 44-50.
- Lewis, D.M., Park, K.M., Tang, V., Xu, Y., Pak, K., Eisinger-Mathason, T.S., Simon, M.C., Gerecht, S., (2016) Intratumoral oxygen gradients mediate sarcoma cell invasion. *Proceedings of the National Academy of Sciences of the United States of America* 113, 9292-9297.
- Li, C., Chaung, W., Mozayan, C., Chabra, R., Wang, P., Narayan, R.K., (2016) A New Approach for On-Demand Generation of Various Oxygen Tensions for In Vitro Hypoxia Models. *PloS one* 11, e0155921.
- Liu, L., Simon, M.C., (2004) Regulation of transcription and translation by hypoxia. *Cancer biology & therapy* 3, 492-497.
- Millonig, G., Hegedusch, S., Becker, L., Seitz, H.K., Schuppan, D., Mueller, S., (2009) Hypoxia-inducible factor 1 alpha under rapid enzymatic hypoxia: cells sense decrements of oxygen but not hypoxia per se. *Free radical biology & medicine* 46, 182-191.
- Mueller, S., Millonig, G., Waite, G.N., (2009) The GOX/CAT system: a novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture. *Advances in medical sciences* 54, 121-135.

- Pal, P., Datta, S., Bhattacharya, P., (2000) Studies on the modeling and simulation of a sequential bienzymatic reaction system immobilized in emulsion liquid membrane. *Biochemical engineering journal* 5, 89-100.
- Pandey, P., Singh, S.P., Arya, S.K., Gupta, V., Datta, M., Singh, S., Malhotra, B.D., (2007) Application of thiolated gold nanoparticles for the enhancement of glucose oxidase activity. *Langmuir : the ACS journal of surfaces and colloids* 23, 3333-3337.
- Park, K.M., Blatchley, M.R., Gerecht, S., (2014) The design of dextran-based hypoxia-inducible hydrogels via in situ oxygen-consuming reaction. *Macromolecular rapid communications* 35, 1968-1975.
- Park, K.M., Gerecht, S., (2014) Hypoxia-inducible hydrogels. *Nature communications* 5, 4075.
- Peng, C.C., Liao, W.H., Chen, Y.H., Wu, C.Y., Tung, Y.C., (2013) A microfluidic cell culture array with various oxygen tensions. *Lab on a chip* 13, 3239-3245.
- Rajan, N., Narayan, A., Wu, Z., Wu, P., Ahn, C.H., Narayan, R.K., Li, C., (2013) A novel oxygen tension programmable microfluidic system (oPROMs) for in vitro cell biology studies. *2013 Transducers & Eurosensors XXVII: The 17th International Conference on Solid-State Sensors, Actuators and Microsystems (TRANSDUCERS & EUROSENSORS XXVII)*, pp. 412-415.
- Semenza, G.L., (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *Journal of applied physiology* 88, 1474-1480.
- Simon, M.C., Keith, B., (2008) The role of oxygen availability in embryonic development and stem cell function. *Nature reviews. Molecular cell biology* 9, 285-296.
- Sobotta, M.C., Barata, A.G., Schmidt, U., Mueller, S., Millionig, G., Dick, T.P., (2013) Exposing cells to H₂O₂: a quantitative comparison between continuous low-dose and one-time high-dose treatments. *Free radical biology & medicine* 60, 325-335.

- Trachootham, D., Alexandre, J., Huang, P., (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nature reviews. Drug discovery* 8, 579-591.
- Tse, P.H., Gough, D.A., (1987) Time-dependent inactivation of immobilized glucose oxidase and catalase. *Biotechnology and bioengineering* 29, 705-713.
- Wu, Q., Wang, L., Yu, H., Wang, J., Chen, Z., (2011) Organization of glucose-responsive systems and their properties. *Chemical reviews* 111, 7855-7875.
- Wykoff, C.C., Beasley, N.J.P., Watson, P.H., Turner, K.J., Pastorek, J., Sibtain, A., Wilson, G.D., Turley, H., Talks, K.L., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., Harris, A.L., (2000) Hypoxia-inducible Expression of Tumor-associated Carbonic Anhydrases. *Cancer Research* 60, 7075-7083.
- Zitta, K., Meybohm, P., Bein, B., Huang, Y., Heinrich, C., Scholz, J., Steinfath, M., Albrecht, M., (2012) Salicylic acid induces apoptosis in colon carcinoma cells grown in-vitro: influence of oxygen and salicylic acid concentration. *Experimental cell research* 318, 828-834.

Figure Captions

Figure 1. Enzyme reaction mechanisms and soluble GOX induced solution hypoxia. (A)

Reaction equilibrium of GOX (i), CAT (ii), and combined reaction (iii). (B, C) GOX-induced O₂ consumption in the absence (B) or presence of 9.8 µg/mL CAT (C). All reactions were carried out in pH 7.4 PBS with constant stirring, at room temperature, and with 25 mM β-D-Glucose. (Mean ± SEM, n ≥ 3).

Figure 2. Effect of GOX modification on oxygen consumption. (A) Reaction scheme of GOX

modification using Acryl-PEG-SVA. Protein structure for GOX was obtained from the RCSB Protein Data Bank (PDB-ID, 3QVP). (B) O₂ consumption profile using soluble GOX or GOX_{PEGA}, 9.8 µg/mL CAT, and 25mM β-D-Glucose. (C) Reaction velocity of O₂ consumption by GOX or GOX_{PEGA} as a function of substrate β-D-glucose concentration. Values were generated from using 0.260 µM GOX or GOX_{PEGA} with 0.30 to 25 mM of β-D-glucose. All reactions were carried out in pH 7.4 PBS with constant stirring at 25°C. (Mean ± SEM, n ≥ 3).

Figure 3. Effect of GOX modification on oxygen consumption over extended time. (A, B)

O₂ consumption profiles of unmodified and acrylated GOX in the absence (B) or presence of soluble CAT (450 µg/mL). All reactions were carried out in pH 7.4 PBS, at 25°C, with 25 mM β-D-Glucose. (**p < 0.01. ***p < 0.001. Mean ± SEM, n ≥ 3).

Figure 4. Effect of hydrogel-immobilized GOX_{PEGA} on oxygen consumption. (A) Schematic

of O₂ measurement within and outside of a PEGDA hydrogel. The sensor probe was fully extended from the needle for measuring O₂ tension exterior to the hydrogel (left). To measure O₂ content at the interior of the hydrogel (right), the optic fiber was recessed within its needle housing to prevent damage of the gel matrix to the probe. After penetration the fiber was extended to the tip of the needle cannula so that it was exposed to the interior of the hydrogel.

(B) O₂ consumption at the interior or exterior of GOX-immobilized hydrogels (120 µL of 8 wt% PEGDA gel with 4 mg/mL GOX_{PEGDA}). (***p < 0.001. Mean ± SEM, n ≥ 3).

Figure 5. Effect of supplements on oxygen consumption and H₂O₂ production by GOX_{PEGDA} gels. (A, B) Effect of soluble CAT addition on O₂ tension (A) and H₂O₂ accumulation (B). (C, D) Effect of additional glucose on O₂ tension (C) and H₂O₂ accumulation (D) in the presence of soluble CAT. Additional bolus injections of glucose (50 µL of 500 mM) was delivered 5 minutes before measuring O₂ at 24 and 48 hour time points. Hydrogels (60 µL) were formed by 15 wt% PEGDA co-polymerized with 6 mg/mL GOX_{PEGDA}. All reactions were carried out in pH 7.4 PBS at 37°C. (**p < 0.01. ***p < 0.001. Mean ± SEM, n ≥ 3).

Figure 6. Cytocompatibility of PEGDA hydrogels with or without immobilized GOX_{PEGDA}. Molm14 cell viability (A, C) and density (B, D) when cultured in the absence (i.e., Empty Gel; A & B) or presence (C, D) of immobilized GOX_{PEGDA} Gel + CAT (6 mg/mL). Hydrogels were formed by 15 wt% PEGDA. CAT in media: 0.54 mg/mL (*p < 0.05. ***p < 0.001. Mean ± SEM, n ≥ 3).

Figure 7. Effect of enzyme-induced hypoxia on cell fate *in vitro*. O₂ profile (A, C) and CA9 mRNA expression (B, D) in Molm14 (A, B) or Huh7 cells (C, D) cultured in the presence of a GOX_{PEGDA} (6 mg/mL) immobilized 15 wt% PEGDA hydrogel. CAT in media: 0.54 mg/mL. CoCl₂ (150 µM) was added separately as an additional control group (*p < 0.05, **p < 0.01, ***p < 0.001. Mean ± SEM, n ≥ 3).

Figure 1.

A.

B.

Figure 2.

Figure 3.

Figure 4.

A.

B.

Figure 5.

Figure 6

A.

B.

C.

D.

Figure 7.

A.

B.

C.

D.

Table

Table 1. Michaelis-Menten constants of GOX and GOX_{PEGA}.

	$V_{\max}$ (mM·min ⁻¹)	K_m (mM)
GOX	0.88 ± 0.05	4.38 ± 0.90
GOX _{PEGA}	0.66 ± 0.03	1.17 ± 0.28