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Oxygen producing gellan gum hydrogels for dual delivery of either oxygen or peroxide with doxorubicin

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Abstract

Hypoxic environments in the core of tumors can give rise to resistance against anti-cancer therapeutics. Oxygen producing biomaterials may be able to improve chemotherapeutic efficiency by locally disrupting the hypoxic environment. We hypothesized that gellan gum hydrogels could be loaded with both a solid peroxide and the chemotherapeutic drug doxorubicin, to release both oxygen and doxorubicin simultaneously. We show that calcium peroxide physically crosslinks gellan gum into a hydrogel which, when loaded with catalase, raises the dissolved oxygen content of media for up to 64 hours. Additionally doxorubicin could be loaded into the hydrogel *in situ*, allowing release in well-defined quantities.

Main Text

Gellan gum is an FDA approved food additive that has more recently been analyzed for hydrogel applications in tissue engineering and regenerative medicine. These applications include bone regeneration,¹ cartilage repair,² intervertebral disc regeneration,³⁻⁴ neuronal cell culture⁵⁻⁷ and spinal cord injury therapies.⁸ The divalent calcium cation derived from calcium chloride is typically used to physically crosslink gellan gum into a hydrogel structure.⁹ From this starting point, we wanted to assess whether calcium peroxide could be used instead of calcium chloride, to form physically crosslinked hydrogels capable of releasing oxygen to cells in culture without causing toxicity.

Oxygen producing biomaterials are a relatively recently developed phenomenon, which have largely focused on the incorporation of solid peroxides for applications in tissue engineering¹⁰⁻¹¹ or cell transplantation.¹² The solid peroxide relies on the presence of water to produce hydrogen peroxide as an intermediate followed by water and oxygen.¹² Other oxygen producing materials have encapsulated liquid hydrogen peroxide¹³, but both methods rely on a catalyst (commonly catalase) to ensure complete conversion to the final oxygen product without peroxide induced toxicity. For biological applications solid peroxides must be encapsulated in a surrounding matrix because a basic by-product is formed during the reaction ($\text{Ca}(\text{OH})_2$ in the case of calcium peroxide). Encapsulating materials have consisted of polydimethylsiloxane (PDMS),¹² methacrylated gelatin¹⁴ or poly(d,l-lactide-co-glycolide).¹⁰⁻¹¹ These materials effectively hold the solid peroxide and byproduct in place and can limit water diffusion to the peroxide to try and slow the oxygen release rate.

To date, the natural polysaccharide gellan gum has not been investigated for encapsulating a solid peroxide for oxygen delivery. Additionally, oxygen producing biomaterials for use in anticancer applications has been largely overlooked. Since hypoxia plays an important role in cancer drug resistance,¹⁵⁻¹⁶ oxygen producing materials may potentially be effective at disrupting the hypoxic environment of a tumor core. We were therefore interested to see if gellan gum hydrogels could also be used to simultaneously deliver oxygen and the chemotherapeutic drug doxorubicin.

The aim of this study was to create a dual delivery system that could either deliver oxygen or peroxide in conjunction with a model anti-cancer drug, doxorubicin. We hypothesized that calcium peroxide could be used to physically crosslink gellan gum into a hydrogel instead of the commonly used calcium chloride. Additionally, we hypothesized that doxorubicin could be incorporated during the gelation process and be released in defined quantities. The specific objectives of this study were to compare gellan gum hydrogels crosslinked with calcium chloride (termed control) and calcium peroxide (termed oxyGG) in terms of rheological properties and oxygen production. The cytotoxicity of oxyGG with and without catalase and with and without prior loading of doxorubicin was also performed.

Four oxygen producing gellan gum hydrogels (oxyGG)(always 1% w/v) were produced using varying degrees of calcium peroxide content (w/v) from 0.1% (oxyGG 0.1%), 0.5% (oxyGG 0.5%), 1% (oxyGG 1%) and 2% (oxyGG 2%). Since divalent cations are required for the physical gelation of gellan gum, control hydrogels were produced using 0.5% w/v calcium chloride (CaCl₂). The hydrogels consisted of gellan gum (Gelzan™ CM, Sigma) dissolved in deionized water at 90°C. After cooling to 60°C, HEPES buffer and HCl were added to maintain a pH of 7.4 upon the addition of calcium peroxide. Catalase (10 units per gel) was then added and lastly the calcium peroxide was added immediately prior to hydrogel casting. The reaction solution formed a hydrogel upon cooling to room temperature in a cylindrical mold of 8 mm in diameter and 1 mm deep (detailed methods are available in the supporting information). The schematic diagram in **figure 1** shows a depiction of the five component system which relies on the influx of aqueous solution (in this case phosphate buffered saline (PBS) or cell culture media) to react with the calcium peroxide to produce hydrogen peroxide and in turn oxygen. The presence of catalase within the hydrogel ensures the hydrogen peroxide is converted to molecular oxygen and the water byproduct, and is essential of maintaining high cellular viability.

The hydrogel is formed via Ca²⁺ ions physically crosslinking the gellan gum network. The stiffness of the hydrogel networks (G' in **figure 1c** and **supporting information figure S1**) was measured by rheology and it was shown that all hydrogels formed from CaO₂ were much less stiff than those formed from 0.5% w/v CaCl₂. The lower solubility of CaO₂ (only 1.72g/L at 20°C) results in less free Ca²⁺ ions for crosslinking the gel. Simply cooling gellan gum back to room temperature without the addition of either CaO₂ or CaCl₂ did not result in hydrogel formation. Upon analysis of the structure of the dehydrated gels by scanning electron microscopy (SEM), a distinct difference could be observed between those gels that contained calcium peroxide and those containing CaCl₂ (**figure 1b**). The difference in surface morphology was particularly apparent for the highest amount of calcium peroxide tested (2% w/v), which showed a highly porous structure, presumably due to the large amount of gaseous oxygen produced throughout the structure. This change in structure could account for the reduced stiffness of the oxyGG 0.5% hydrogels compared to the hydrogels formed with 0.5% w/v CaCl₂.

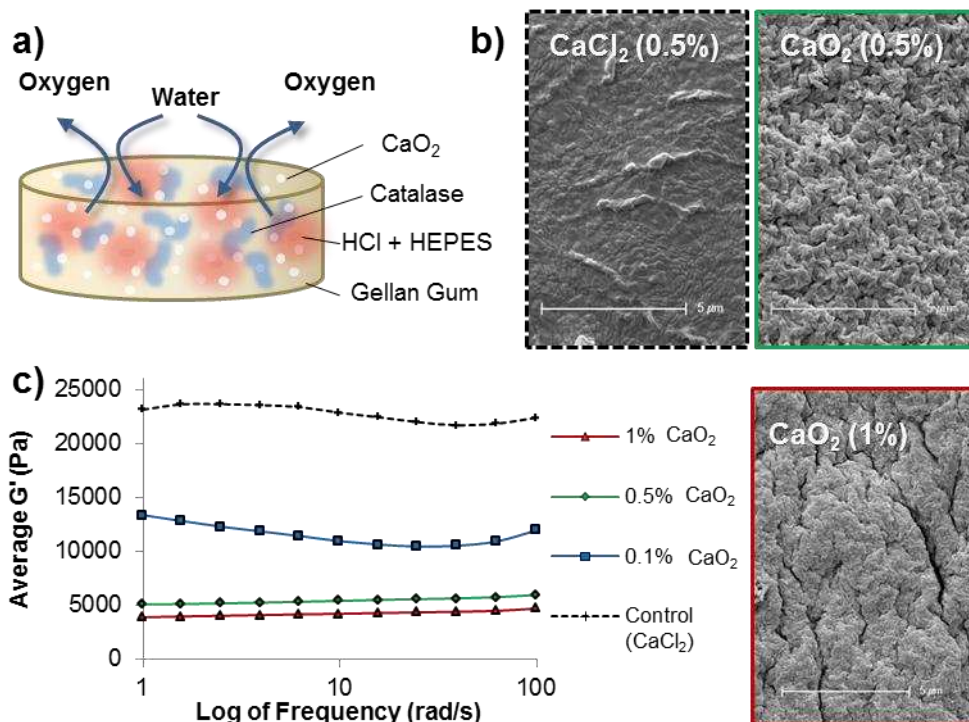


Figure 1. Oxygen producing gellan gum hydrogels have variable stiffness. A schematic depiction (a) of the multi-component hydrogel, containing gellan gum, calcium peroxide (CaO_2), and catalase which is neutralized and buffered by hydrochloric acid and HEPES buffer respectively. SEM images (b) of control hydrogels where calcium chloride is used instead of calcium peroxide (CaCl_2 (0.5% wt/vol)), in comparison with the oxygen producing CaO_2 0.5% w/v, and 1% w/v hydrogels showing the change in morphology due to oxygen production. Rheological analysis (c) of the different gel compositions showing that control hydrogels with CaCl_2 (0.5% w/v) are stiffer than all other formulations ($n=5$, error bars removed for clarity). See supporting information for further SEM and rheological analysis.

Oxygen production was analyzed for hydrogels composed of 0.1%, 0.5% and 1% calcium peroxide using a PreSens non-invasive oxygen sensor. Catalase is used for the decomposition of H_2O_2 via the $\text{Fe}^{2+}/\text{Fe}^{3+}$ within the catalase, to avoid unwanted side reactions and cellular damage.¹⁷ Catalase was incorporated directly into the gellan gum hydrogel during gelation and was not added to the surrounding medium. Oxygen production was analyzed in open cuvettes in both normoxic and hypoxic conditions, by first allowing PBS to equilibrate before adding the oxyGG hydrogel disks into the cuvette. The oxygen release profile was clearly dependent on the amount of calcium peroxide within the hydrogel, and was similar for both conditions, but with greater values reached in normoxia (figure 2). In normoxic conditions the oxyGG (0.5%) caused a 220% rise in dissolved oxygen content, therefore more than double that of PBS left in atmospheric conditions. The oxyGG (1%) hydrogel caused an even greater increase in oxygenation (485% rise). The oxyGG (0.5%) and oxyGG (1%) hydrogels resulted in oxygen levels that stayed elevated above the PBS control for between 24 and 64 hours, and both were longer above the PBS control baseline in hypoxic conditions. The pH of the PBS was analyzed over the entire release period in order to check that the hydrogels buffered with HEPES did not cause a rise in pH over time. Unlike pure calcium peroxide that caused a rise in pH to 10.7 over 72 hours, oxyGG 0.1%, 0.5% and 1% cause no increase in pH over this time (see supporting information figure S2). These data show that oxyGG hydrogels can deliver large amounts of oxygen into solution for two days without causing a rise in pH. The level of oxygen in tumors is highly variable but to give some context for the oxygen production herein, primary brain tumors have an oxygen partial pressure of 13 mmHg (\approx

0.718 mg/L O₂)(normal brain is estimated at 35 mmHg (\approx 1.933 mg/L O₂).¹⁸ It is clear that the rise in dissolved oxygen to 15mg/mL by the oxyGG (0.5%) formulation under extreme hypoxic conditions represents a large oxygen production, and so was used for further studies for cytotoxicity analysis and oxygen delivery *in vitro*.

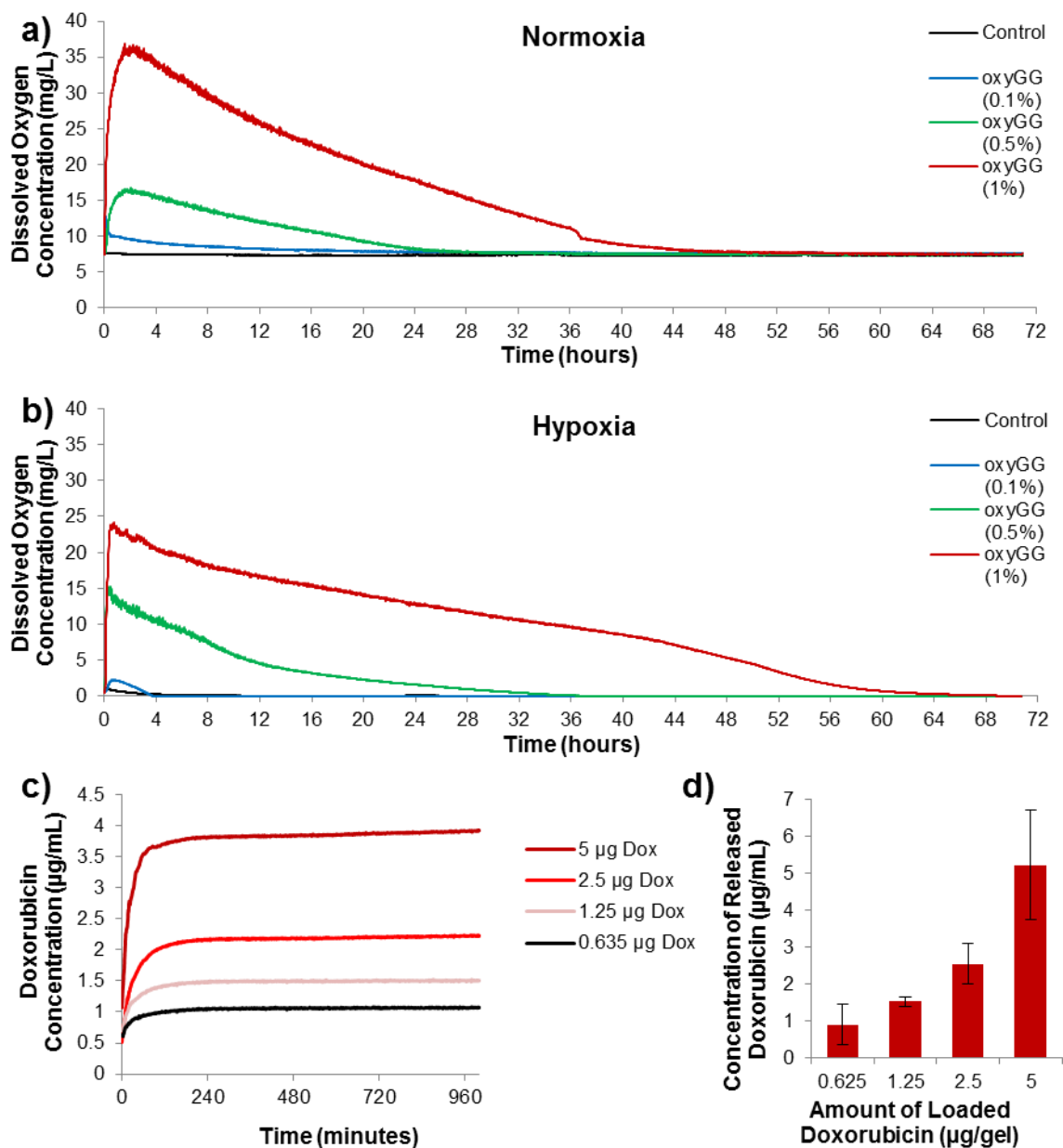


Figure 2. Tunable release of either oxygen or doxorubicin can be achieved from gellan gum hydrogels. Oxygen release profiles of oxygen producing gellan gum hydrogels (oxyGG) with varying percentage (w/v) of calcium peroxide (indicated in brackets), showing that the oxygen release profile can be tuned by varying the calcium peroxide content. The oxygen release in normoxic conditions (a) shows higher peak oxygen values for all groups than when the oxyGG hydrogels are analyzed in severe hypoxic conditions (0.1% oxygen) (b). The control group is a hydrogel formed with calcium chloride for both analyses. Doxorubicin is released from the hydrogels over a period of \sim 2 hours, as shown in a representative release curve (c) and the final concentration closely corresponds to the initial amount loaded in the hydrogels (d) ($n=3$, error bars represent \pm standard deviation). For oxygen release curves for oxyGG hydrogels without catalase please see supporting information figure S8 and S9.

Aside from the predominant mechanism of doxorubicin action (intercalating with DNA base pairs causing DNA damage (including mitochondrial DNA)),¹⁹ doxorubicin can also bind cell membrane proteins directly causing the production of highly reactive species of hydroxyl radicals.²⁰ Hypoxia has been shown to reduce the cytotoxic action of doxorubicin via HIF related pathways, the induction of MAX dimerization protein 1 (Mad1)²¹ or the induction of the multidrug resistance-associated protein 1 gene (MRP1).²² It was therefore desired to analyze whether doxorubicin could be loaded to the oxyGG hydrogels during gelation and what amount of it would be released into the surrounding media. Hydrogels were formed with different amounts of doxorubicin varying from 0.635 μg to 5 μg and placed into 1 mL of phosphate buffered saline (PBS) and kept at 37°C in a sealed cuvette. Cumulative doxorubicin release was measured by UV absorbance at 490 nm for 1000 minutes. An example set of release curves are shown in **figure 2c**. The majority of the doxorubicin is released from the hydrogels in the first two hours of incubation, probably due to the inert and uncharged polysaccharide structure of gellan gum. The final concentrations from three such experiments were averaged and plotted in **figure 2d** which shows that the amount of doxorubicin released corresponds almost exactly to the amount loaded during gelation.

With the capacity for dual delivery of both oxygen and doxorubicin we wanted to see if the high amount of oxygen released from the oxyGG hydrogels could improve the action of doxorubicin against C6 cells *in vitro*. C6 cells are a rat glioblastoma cell line which have previously been used to study the induction of hydroxyl radical (HO^\bullet) due to treatment with doxorubicin.²³ However, first we analyzed whether the oxyGG hydrogels would be inherently toxic due to the high level of oxygen production. For this study, primary astrocytes extracted from the newborn rat²⁴ were used as a test platform to represent a predominant cell type of the brain. Toxicity analysis was performed using a combination of the PrestoBlue assay (which measures cell metabolic activity), the Quant-iT PicoGreen assay (which measures total DNA content) and light microscopy. Interestingly the cell metabolic activity was far higher for astrocytes that are incubated with the oxyGG hydrogels than of those without (hypoxic conditions - **figure 3a/ supporting information figure S3**, and normoxic conditions - **supporting information figure S4**). Since this strong effect was present even after one day, an increase in proliferation was unlikely. Indeed, the total DNA content of the wells was not increased, but instead was slightly decreased for the oxyGG treatment group for all time points except day 6 (**figure 3b**). Microscopy analysis generally showed little or no changes in cell morphology between the groups except that a few patches of cells containing extended processes could be observed in the oxyGG group (**figure 3c**).

Having established that the oxyGG hydrogels appear nontoxic to astrocytes, the dual delivery study with C6 was carried out in both hypoxic conditions (**figure 3d-f/ supporting information figure S5**) and normoxic conditions (**supporting information figure S6**). The toxicity of various oxyGG formulations was carried out in comparison to untreated C6 cells and doxorubicin treated cells ("Free Dox" group – a concentration of 4 $\mu\text{g}/\text{mL}$ was used which has previously been shown to be quite toxic to a variety of cancer cells in culture²⁵). Formulations were also made without catalase (oxyGG – cat) with doxorubicin (+ dox) and without doxorubicin (– dox). Similarly to the astrocyte experiment oxyGG (henceforward termed oxyGG + cat) caused a significant rise in metabolic activity (**figure 3d**), reduced total DNA content (**figure 3e**) but did not affect cell morphology (**figure 3f**). Doxorubicin added to the media reduced the cellular metabolic activity to 33% after 1 day in culture. A far larger decrease could be obtained by both formulations of oxyGG without catalase indicating that they are releasing the reaction intermediate hydrogen peroxide to the cells. By the second and

third day, the differences in metabolic activity between the free doxorubicin and the oxyGG – cat formulations were smaller. No difference in total DNA content (**figure 3e**) was observed between doxorubicin and the dual delivery groups.

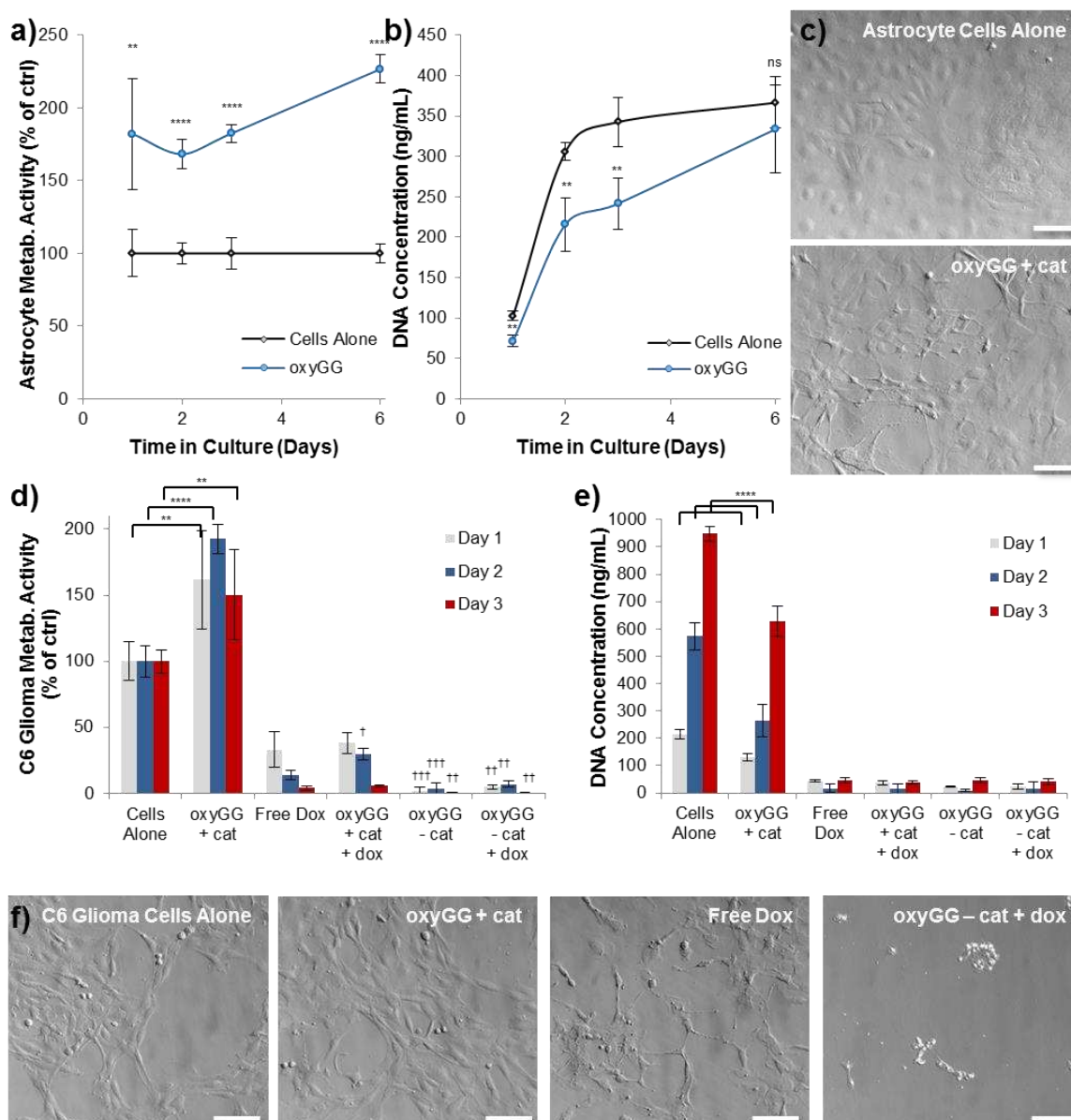


Figure 3. *In vitro* analysis of the cytotoxicity of various formulations of calcium peroxide crosslinked gellan gum (oxyGG). Astrocyte cell metabolic activity (**a**) is increased by the addition of oxyGG, whilst the total DNA content is reduced until day 6 (**b**). Light microscope images of each time point are shown in the **supporting information figure S3** with part (**c**) above showing untreated astrocytes (top) and oxyGG treated cells (bottom). The same trend is shown for C6 glioma (**d** + **e**) and the addition of free doxorubicin vastly reduces the cellular viability. Hydrogels formed without catalase (oxyGG – cat) show a further reduction in cell metabolic activity at all time points due to hydrogen peroxide release (**supporting information figure S10**). Light microscope images (**f**) show C6 glioma exposed to various treatment groups. All scale bars represent 100 μ m, $n=4$ for all experiments, error bars represent \pm standard deviation. Asterisks (*) represent a statistically significant difference between cells alone and oxyGG hydrogels (unpaired student t test), whilst daggers (†) represent a statistically significant difference between cells alone and free doxorubicin (unpaired student t test).

represents statistically significant differences between free doxorubicin and the other toxic treatment groups (one way ANOVA, with Tukey's multiple comparisons test).

We had hypothesized that the additional oxygen generated by the oxyGG could enhance the toxicity of the doxorubicin. However, the cytotoxic effect of oxyGG + cat + dox group was either not different to that of the free doxorubicin group (day 1 and day 3) or even slightly less toxic (day 2). We have instead shown that oxyGG hydrogels without catalase are highly effective at killing C6 glioma cells *in vitro* due to the lack of conversion of hydrogen peroxide to oxygen. An additive effect of the dual delivery of both peroxide and doxorubicin was unfortunately impossible to observe since the oxyGG – cat hydrogels completely killed all the cells even after one day in culture.

Due to the highly undesirable side effects of chemotherapy, there is currently much research into better delivery of chemotherapeutic agents. Biomaterial delivery systems such as nanotubes,²⁶⁻²⁷ nanoparticles,²⁸⁻²⁹ micelles,³⁰ and drug eluting devices³¹ are being developed to reduce the dose required with the aim of reducing side effects and thus improving effectiveness. Herein, our approach has been to develop a material that delivers a defined quantity of drug along with oxygen or peroxide in an attempt to improve the efficiency of chemotherapeutic drug action. Whilst macroscale implants have been used for controlled drug release following brain tumor resection³², downscaling this gellan gum hydrogel for injection would be highly desirable, though the oxygen and drug release profiles would likely differ from the bulk material described herein. Whilst the killing effect of doxorubicin was not enhanced via the addition of oxygen to 2D *in vitro* culture of C6 glioma, it would be interesting to investigate whether additive effects are observed in 3D cancer spheroid models. Spheroid culture within hydrogel matrixes show higher resistance to doxorubicin treatment, even at doses of 4 µg/mL,²⁵ and spheroid cultures have been shown to have hypoxic cores, which are effected by surrounding oxygen levels.³³⁻³⁴ Furthermore, such materials hold the potential to affect the efficiency of more oxygen dependent anti-cancer therapy strategies such as radiotherapy and photo-dynamic therapy, both of which require oxygen as a means of action.³⁵⁻³⁶ Since both radiotherapy and photo-dynamic therapy have been reported to be less effective in oxygen partial pressures of 25-30 mmHg (\approx 1.381 mg/L - 1.657 mg/L O₂) and 15-35 mmHg (\approx 0.892 mg/L - 1.933 mg/L O₂) respectively,³⁷ oxygen producing materials may be able to increase the local oxygen concentration for more effective therapy.

In conclusion we have developed an oxygen producing hydrogel based on the FDA approved natural polysaccharide gellan gum. This hydrogel encapsulates the solid peroxide calcium peroxide, catalase and doxorubicin if required. The oxygen release profile from the hydrogel disks showed elevated oxygen levels for up to 48 hours in normoxic conditions and 64 hours in hypoxic conditions, which was highly dependent on the amount of calcium peroxide embedded. The hydrogels were non-toxic towards C6 rat glioma cells and primary rat astrocytes if cultured in either normoxic or hypoxic conditions. The hydrogels could deliver a well-defined amount of doxorubicin to the C6 cells but the cytotoxic effect was not enhanced by the simultaneous release of oxygen. By omission of catalase during the gelation step, the gellan gum hydrogels become highly toxic due to the lack of conversion of the produced peroxide to oxygen. This work shows the first use of calcium peroxide as a crosslinking agent for gellan gum. By adjusting a variety of parameters such as the calcium peroxide to catalase ratio one could envisage tunable peroxide/oxygen release. Future plans aim to assess the use of these materials in three dimensional *in vitro* models for applications in photodynamic therapy where high oxygen/peroxide production may greatly enhance the therapeutic effect.

Supporting Information

Supporting Information contains experimental method details and additional data.

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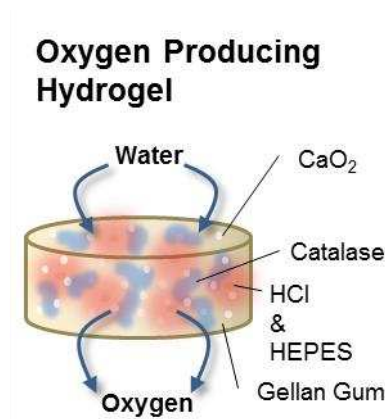
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TOC entry:

Oxygen producing gellan gum hydrogels for dual delivery of either oxygen or peroxide with doxorubicin

Ben Newland, Marcel Baeger, Dimitri Eigel, Heike Newland, and Carsten Werner*



Supporting Information

Oxygen producing gellan gum hydrogels for dual delivery of either oxygen or peroxide with doxorubicin

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Experimental Section

Materials:

Gellan gum (Gelrite®) (henceforth termed GG) and all other reagents were purchased from Sigma Aldrich unless otherwise stated. Doxorubicin was purchased from LC Laboratories, USA. Water used throughout this water was deionized and filtered via the MilliQ ultrapure purifier system.

Preparation of the gellan gum hydrogels:

First 200 mg GG was dissolved in 20 mL water under constant stirring for 30 minutes in order to gain a solid content of 1% (w/v). Next, different amounts of CaCl₂/CaO₂ (Table S1) were added to the solution to adjust for different cross linking degrees. In order to buffer the basic gels to physiological pH (7.4), 0.1 M HEPES was added before the addition of CaO₂ and different amounts of HCl (37%) as a 1.2 molar excess (obtained empirically) was added afterwards and the pH was checked before any further usage. Beginning with the lowest concentration, 2 mL of solution was taken and 100 µL gels were formed in plastic molds (cylindrical, ø8 mm). The gels formed when they were allowed to cool down to room temperature.

Table S1: Production of the different cross linking degrees

Desired solid content of CaCl ₂ /CaO ₂	Initial solution	Mass of CaO ₂ added	Vol. removed for gel formation	Added volume of HCl (37%)
0.1% (w/v)	20 mL 1%GG	20 mg	2 mL	30 μ L
0.5% (w/v)	18 mL 1%GG 0.1% CaCl ₂ /CaO ₂	72 mg	2 mL	108 μ L
1% (w/v)	16 mL 1%GG 0.5% CaCl ₂ /CaO ₂	80 mg	2 mL	119 μ L
2% (w/v)	14 mL 1%GG 1% CaCl ₂ /CaO ₂	140 mg	2 mL	209 μ L

To produce catalase containing gels, a 5 μ L drop of catalase at 2 mg/mL was pipetted into the plastic mold before adding the other gellan gum hydrogel components. OxyGG – cat hydrogels received 5 μ L of water instead (carrier solution for catalase). 1 μ L of a 0.5 mg/mL doxorubicin stock solution (in water with 0.1% DMSO) was added to the plastic mold before addition of the gellan gum to create the oxyGG + dox groups. Experiments with the “free doxorubicin” groups received 1 μ L of the same doxorubicin stock solution directly into the cell culture medium.

Rheological Analysis:

8mm diameter disks of hydrogel were punched from the mold, and placed in PBS prior to rheological measurement. The storage modulus (G') was measured by performing oscillatory shear experiments on an ARES LN2 rotational rheometer (TA Instruments, Eschborn, Germany). The distance between the oscillatory plates was reduced to 90% of the original gel height and then frequency sweeps were performed from 1 to 100 rad/s, at 25 °C with a strain amplitude of 2 %. A minimum of six independent experiments were performed and an average was calculated.

Scanning Electron Microscopy (SEM) Analysis:

The formed GG hydrogels were removed from the molds directly onto specimen stubs, dried in a vacuum oven at 37°C and sputter-coated with gold for 60 seconds (BALZERS SCD 050

Sputter Coater) before imaging. SEM studies were performed using a XL30 ESEM-FEG microscope (Philips) in high vacuum mode using accelerating voltages of 5 kV.

Analysis of the Effect of the GG Hydrogels on pH:

Gels of 0.1%, 0.5% and 1% CaO₂ were made up as described and added to 1 ml of PBS in a 24 well plate. The pH of the PBS was measured over three days using a VARIO[®] Cond pH meter. The equivalent mass of CaO₂ which corresponds to a 1% CaO₂ hydrogel was used as a positive control.

Measurement of Oxygen Release:

In order to measure the oxygen production of the CaO₂ cross-linked gels, the oxygen sensor (Presens Oxy-4) was firstly calibrated. The 0% point was set by measuring the O₂ level of a 1% (w/v) sodium sulfite aqueous solution and the 100% point was set by measuring water which was oxygenated before by bubbling through air for 30 minutes. The medium in which the gels were analyzed was 700 µL PBS which was added to the measuring vial (fitted with a Presens spot sensor) one hour before addition of the gel to allow equilibrium to be reached. The oxyGG hydrogels were added directly into the measuring vial and analyzed by taking measurements every 5 minutes for 72 hours. Studies in severe hypoxia were performed via the same set up as described above, but carried out within a hypoxia chamber (Biospherix) set to 0.1% oxygen held at 37 °C.

Analysis of Doxorubicin Release:

Doxorubicin release from the oxyGG hydrogels (0.5% CaO₂) was performed by incubating the samples in 1 mL of PBS at 37°C. Cumulative drug release was measured using the doxorubicin-associated UV absorbance at 490 nm (Beckman Coulter, DU800, USA) at intervals of 15 minutes. oxyGG hydrogels without doxorubicin were used to establish an

absorbance reference value which was subtracted from all readings. Three experiments were performed and the average value for each concentration of loaded doxorubicin was plotted.

Cell Culture and Cell Viability Analysis:

Primary cortical astrocytes were extracted from newborn rat brains as previously described¹, and C6 glioma cells (rat) were purchased from Sigma Aldrich. DMEM + GlutaMax (Gibco) was used for the culture of C6 cells and DMEM/F12 (Gibco) for the astrocyte cells. Both types of media were supplemented with 10% fetal bovine serum (FBS), 0.5% Streptomycin and 0.5% Penicillin. The cells were cultured in T75 culture flasks for 3 / 7 days (C6/Astrocytes resectively) in a CO₂ incubator (CO₂ level: 5%; O₂ level: 21%; 37°C) until usage for the experiments. 24-well plates were seeded with 10,000 cells per well (C6 glioma) or 20,000 cells per well (astrocytes) and were incubated for 24 hours in normoxic or hypoxic conditions (Biospherix hypoxia chamber set to 0.1% oxygen) to adhere.

To analyze the toxicity of the gellan gum hydrogels with varying compositions (i.e. with catalase (+ cat), without catalase (- cat), with doxorubicin (+ dox) or without doxorubicin (- dox) the 8mm hydrogel disks were placed into the pre-seeded wells. After 24 hours of incubation in either normoxic (21% oxygen) or hypoxic (0.1% oxygen), light microscopy images were taken (Olympus), then the hydrogels and the media were removed and replaced with a 10% PrestoBlue solution in media. After 1 hour incubation 100 µl was transferred to a 96 well plate for measurement in TECAN plate reader by fluorescence analysis (excitation wl: 540 nm; emission wl: 590 nm; gain: 70; 3 flashes; lag time: 0µs; integration time: 20µs; room temperature). Four replicates were performed and the measurement for blank PrestoBlue (see **supporting information Figure S7** for variance) was subtracted and the results were analyzed with MS Excel and the cell viability was plotted as a percentage of the control group of cells receiving no hydrogel. Separate well plates were seeded for each time point of the experiment and analyzed as described for the 24 hour incubation.

Analysis of Hydrogen Peroxide Production:

Gellan gum hydrogels of 0.5 % were made up as described above (without and with 5 μ L of 2 mg/mL of catalase) and directly added to 1 mL of PBS in a 24 well plate (n = 4). Additionally, four gellan gum hydrogels without catalase were placed in wells, which already contained 5 μ L of catalase (2 mg/mL) in 1 mL of PBS. All gels were then incubated at room temperature away from light for 2 hours. Afterwards, 800 μ L of the supernatant was removed from each GG hydrogel sample and transferred into a new 24 well plate. In order to measure the hydrogen peroxide release from the GG hydrogels, a fluorimetric hydrogen peroxide assay kit (Sigma Aldrich (MAK 166)). 50 μ L of the standard was added to a 96 well plate in duplicate. To the same 96 well plate, 50 μ L of the removed supernatant was added in quadruplicate. The supernatant was diluted if necessary in a ratio of 1:1000 with PBS. Subsequently, a so-called Master Mix was prepared, which contained 50 μ L of infra-red peroxidase, 200 μ L (20 units/mL) of horseradish peroxidase and 4.75 mL of assay buffer. 50 μ L of this Master Mix was added to each of the wells (standard, sample and blank) and mixed well, whereby the total volume amount in a well was 100 μ L. After 20 minutes of incubation at room temperature protected from light, the fluorescence intensity was measured using the fluorescence plate reader TECAN GENios (excitation wl: 640 nm; emission wl: 680 nm; gain: 112; 3 flashes; lag time: 0 μ s; integration time: 20 μ s; room temperature). The blank for the assay was the value obtained for the 0 μ M hydrogen peroxide standard. This value was subtracted from all readings. The final hydrogen peroxide release concentration of the GG samples was determined from the hydrogen peroxide standard curve.

Statistical Analysis:

This was performed using GraphPad Prism 5 software, using a student's t test or one way analysis of variance (ANOVA). A statistical significant difference between the groups in a time point is represented by asterisks (*) or daggers (†) on the graph, corresponding to the following P values: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Error bars represent \pm standard deviation.

Supplementary Data:

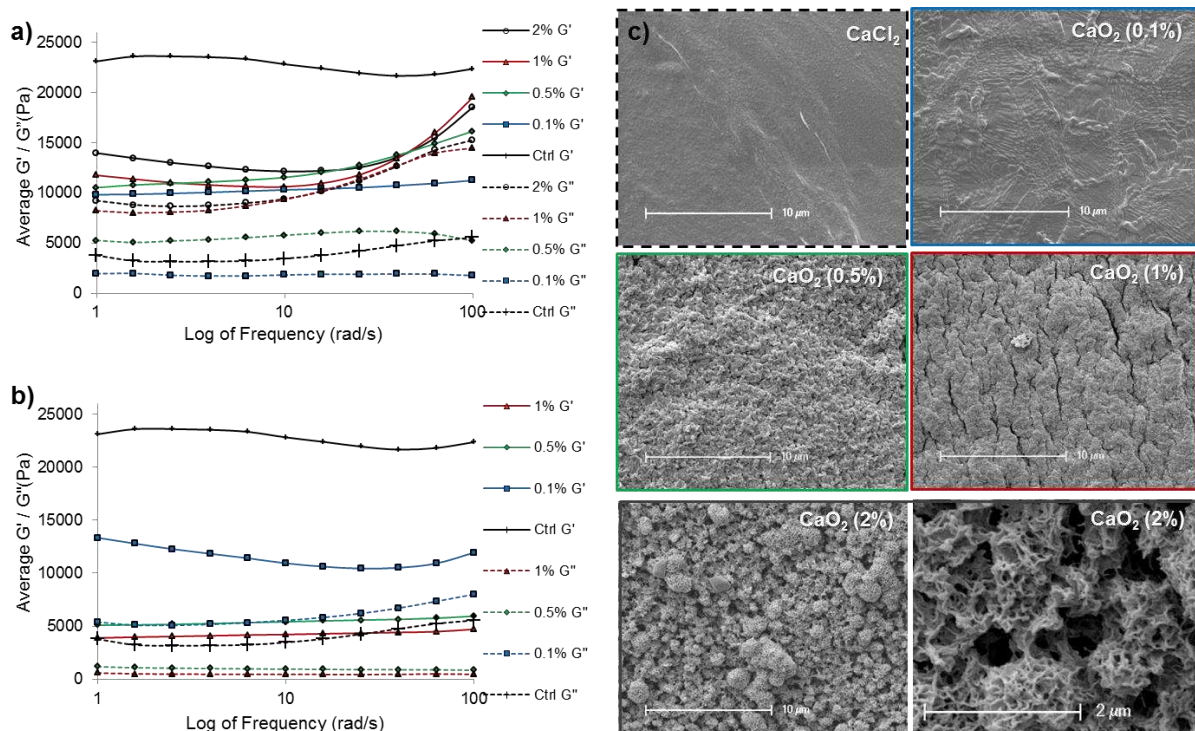


Figure S1 – Rheological analysis of the hydrogels of different compositions after incubation in PBS at 37°C for 24 hours either without being buffered and neutralized (i.e. just CaO_2 added)(a) or buffered (HEPES and HCl added)(b) showing that the buffering process reduces the stiffness of the hydrogels (2% CaO_2 gels were not analyzed as they were too fragile to handle properly). Corresponding scanning electron microscope images (c) of the hydrogel surfaces showing the effect of increasing CaO_2 on surface morphology.

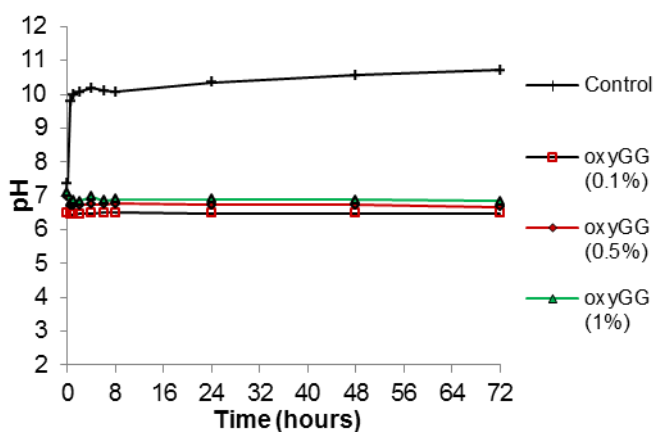


Figure S2 – Analysis of the pH of PBS containing various compositions of oxyGG hydrogels in comparison to calcium peroxide (control), showing that despite the production of oxygen for ~44 hours (Figure 2a), no change in pH can be observed over this period or beyond.

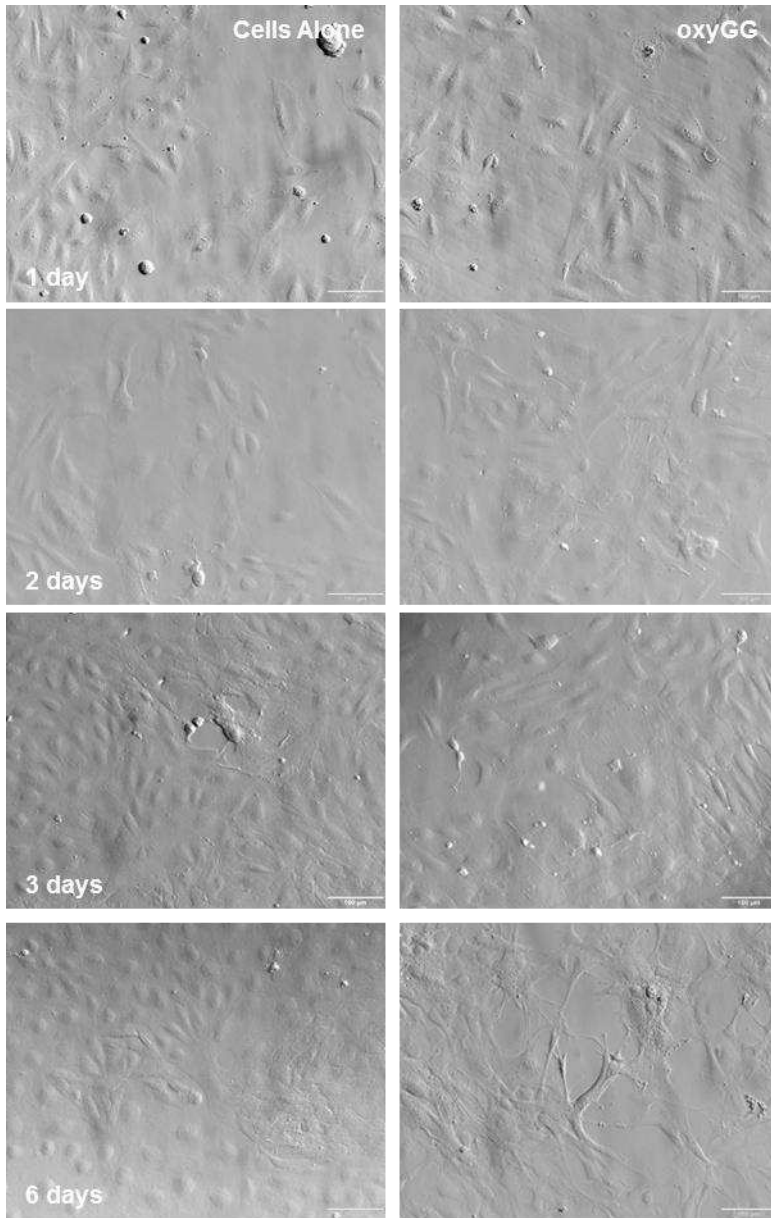


Figure S3 – Representative images of the astrocytes in hypoxic conditions (0.1% oxygen) at each time point with (right hand side) or without (left) oxyGG (scale bars = 100 µm).

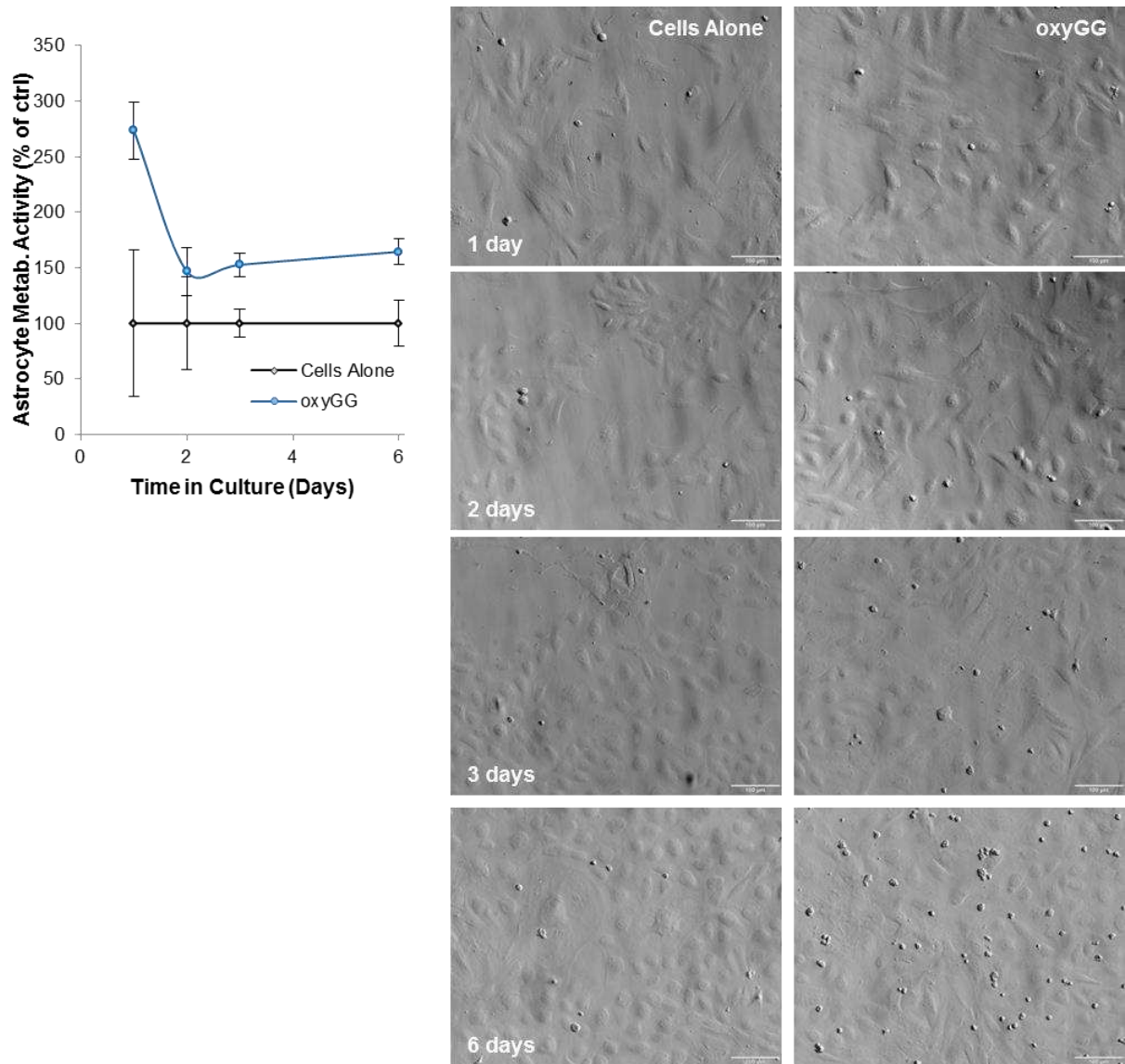


Figure S4 – The metabolic activity of astrocytes cultured with or without oxyGG in normoxic conditions as measured by the PrestoBlue assay (n=4 error bars represent +/- standard deviation). Representative images of the astrocytes in normoxic conditions with or without oxyGG (scale bars =100 μ m).

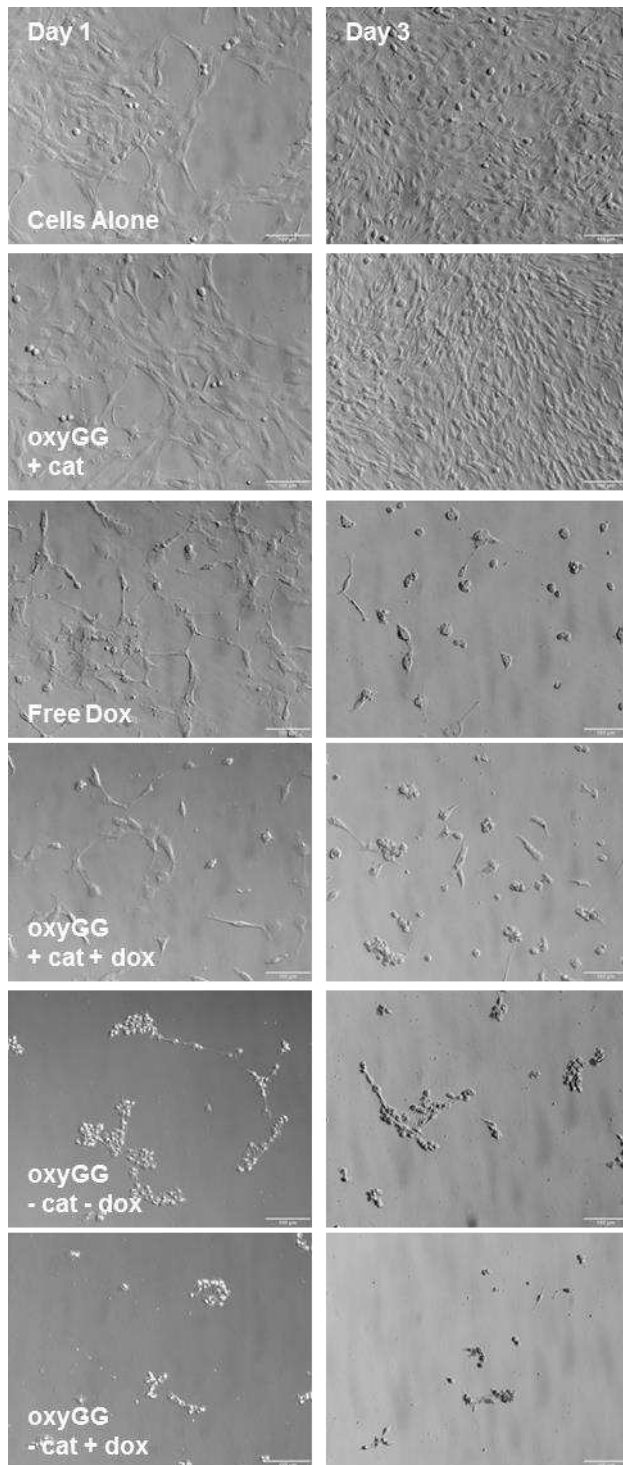


Figure S5 – Representative light microscopy images of C6 glioma cells cultured in hypoxic conditions (0.1% oxygen) with a variety of oxyGG formulations or free doxorubicin in the medium (free dox). (Scale bars = 100 μm)

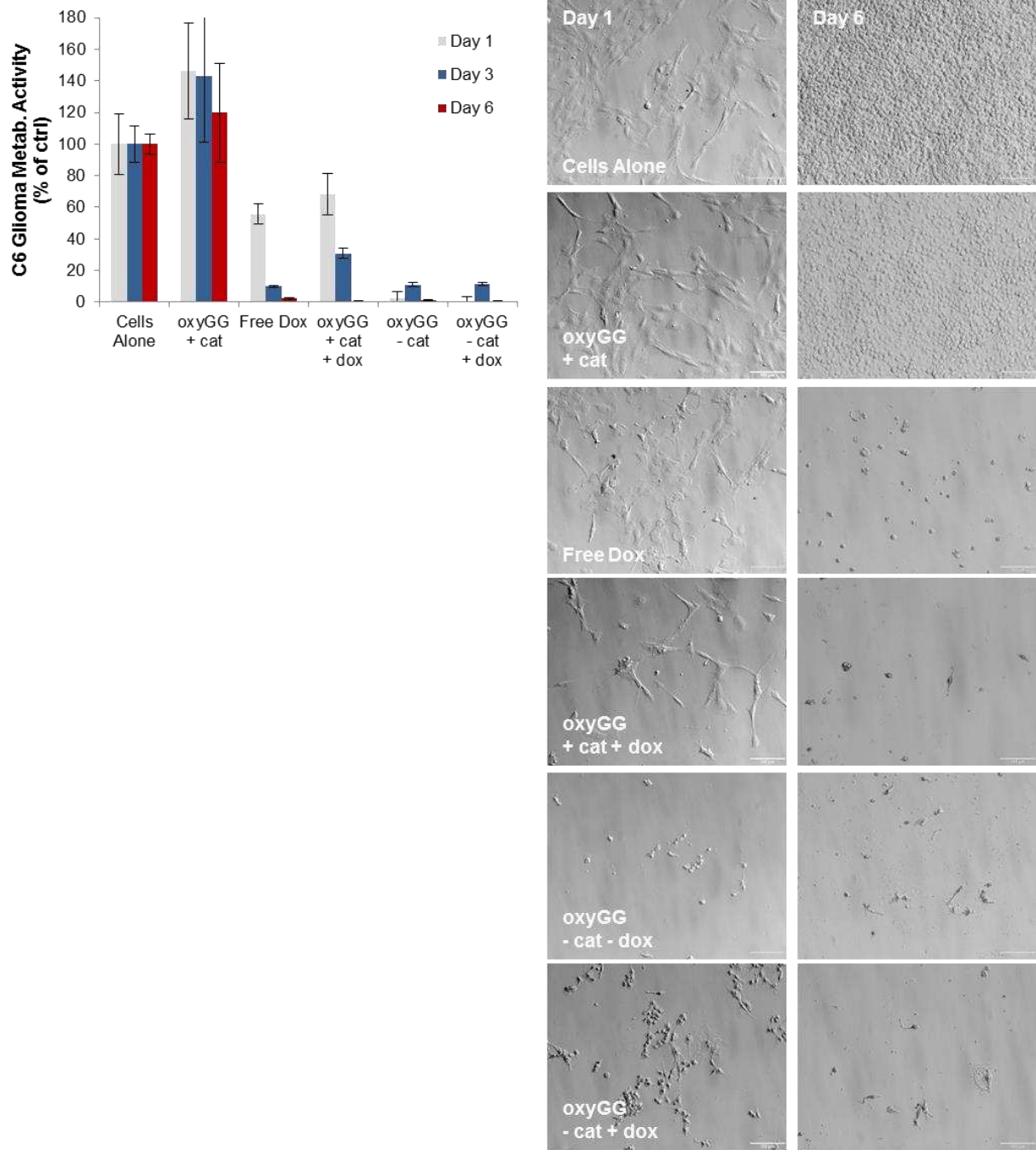


Figure S6 – C6 glioma cell metabolic activity in normoxic conditions after incubation for 1,3 or 6 days with a variety of oxyGG formulations or free doxorubicin in the medium (free dox).

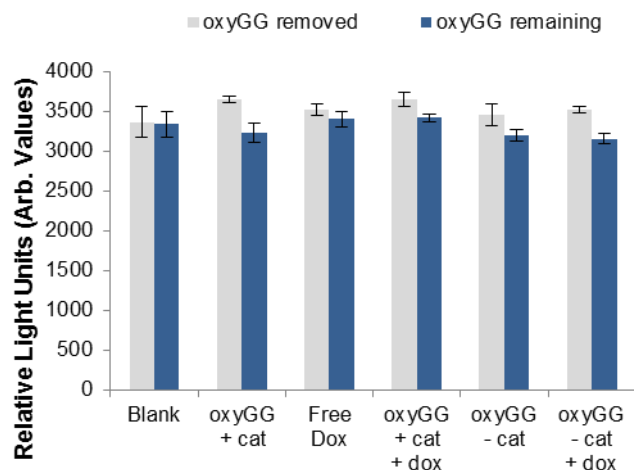


Figure S7 – Analysis of the blank measurements for the PrestoBlue assay for each condition. Neither the catalase, doxorubicin nor the oxyGG effect the blank presto blue reading, whether they were first removed from the well (grey bars) or even left in the wells during the 1 hour PrestoBlue incubation. This shows that they do not interact with the assay to give a false positive/negative.

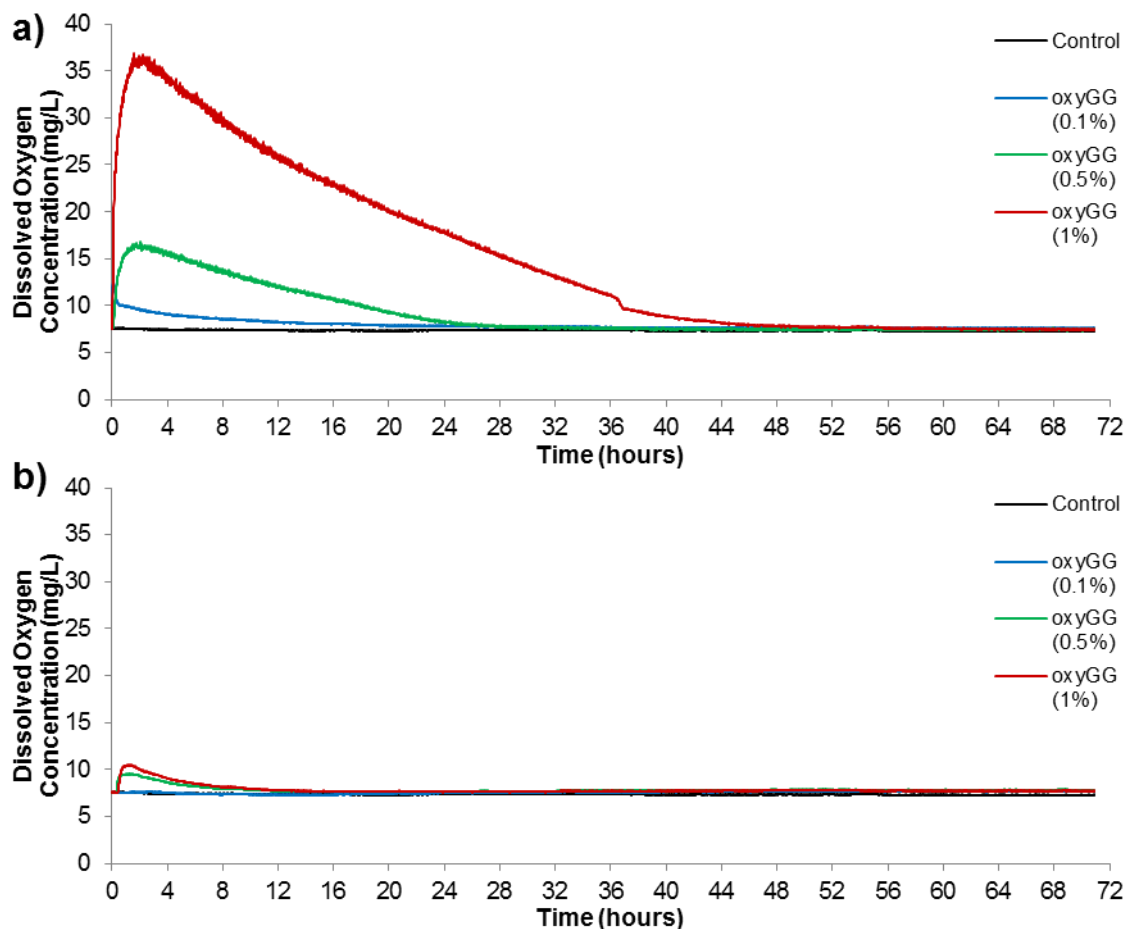


Figure S8 – Oxygen release profile of oxyGG with catalase (a) and without the addition of catalase into the hydrogel (b) in normoxic conditions.

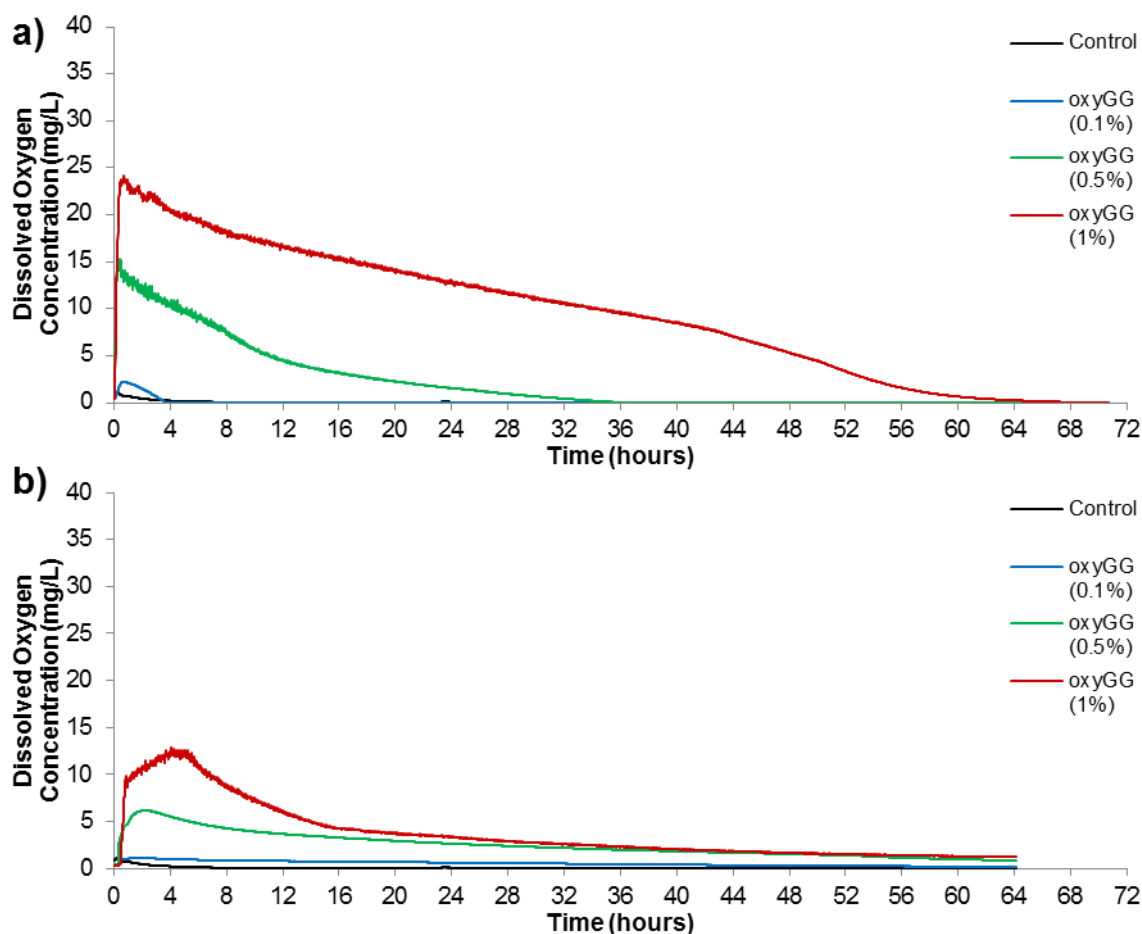


Figure S9 – Oxygen release profile of oxyGG with catalase (a) and without the addition of catalase into the hydrogel (b) under hypoxic conditions.

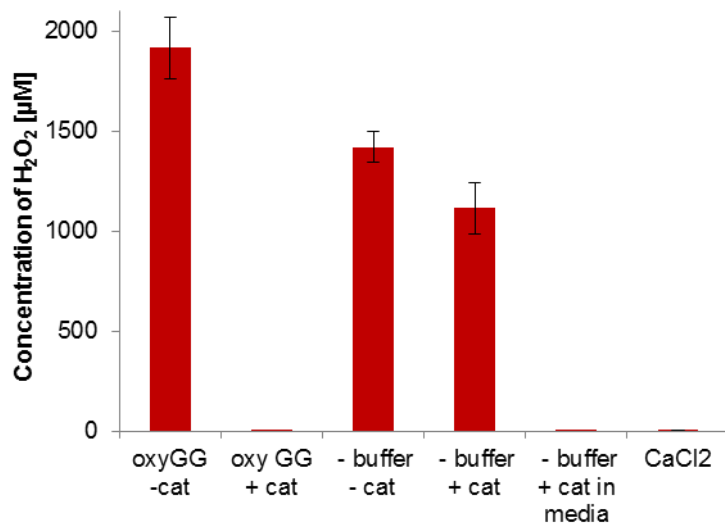


Figure S10 – oxyGG hydrogels formed without catalase cause hydrogen peroxide release whether they are buffered/neutralized (oxyGG – cat) or not buffered/neutralized (-buffer – cat). The addition of catalase reduces the hydrogen peroxide to baseline levels (n=4, error bars represent +/- standard deviation).

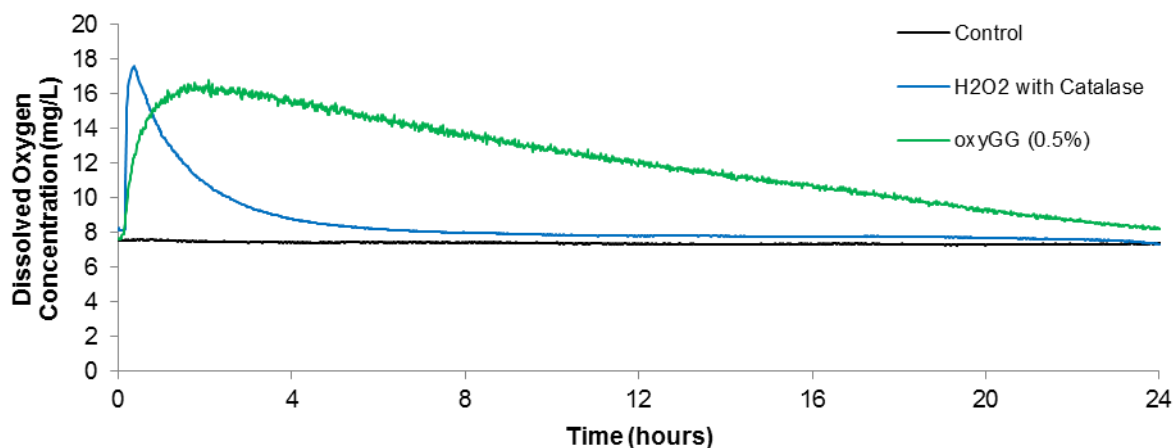


Figure S11 – analysis of the addition of the equivalent amount of hydrogen peroxide to the oxygen sensing cuvette to that theoretically produced by oxyGG (0.5%). Catalase was also added to the media (10 units) to match that contained within oxyGG (0.5%) hydrogels. There is a sudden burst release which drops off quickly as all the H_2O_2 is converted to oxygen and water.

Supporting Information Reference

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