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A Simple and Inexpensive Method to Control Oxygen Concentrations within Physiological and Neoplastic Ranges

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Abstract

Traditional methods for regulating oxygen concentration ($[O_2]$) in *in vitro* experiments over the range found in normal and tumor tissues require the use of expensive equipment to generate controlled gas atmospheres, or the purchase of a range of gas cylinders with certified O_2 percentages. Herein we describe a simple and inexpensive enzymatic method for generating low, precise steady-state $[O_2]$ levels that are stable for several hours. This method is particularly applicable to the *in vitro* study of some classes of hypoxia targeted antitumor prodrugs and bioreductively activated agents.

Introduction

Low $[O_2]$ regions arise in solid tumors from their defective microvascularization which results in an imbalance between O_2 supply and demand (1). These hypoxic regions are a major factor in resistance to radiotherapy since the co-presence of O_2 is required for effective cell kill. Thus, hypoxic tumor cells tend to survive radiation therapy and subsequently cause disease relapse. Strategies to target these radiation resistant subpopulations with hypoxia targeted prodrugs are being actively pursued (1–5). Normal tissue $[O_2]$ is generally between 10–40 μM , while hypoxic tumor cell populations exist in regions with $[O_2]$ levels ranging from ~5 μM down to < 1 μM . Hypoxia targeted prodrugs are usually composed of three domains, a trigger region, a linker region and a therapeutic domain. The trigger is an O_2 sensing domain that typically undergoes one electron reduction to form a radical species by enzymes such as cytochrome P450 reductase (1, 2). The reduced prodrug radical is briskly oxidized by O_2 back to the parental agent. Thus, further reduction, resulting in linker fragmentation and the release of the therapeutic agent, only occurs readily at low $[O_2]$. The affinity of the initial radical product for O_2 determines the sensitivity for O_2 inhibition of prodrug activation. A value K_{O_2} can be defined as the $[O_2]$ inhibiting the

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activation of an agent under test conditions by 50%. Ideally, for a tumor targeted agent, the K_{O_2} value should be sufficiently lower than normal physiological O_2 levels to minimize toxicity to normal cells, while resulting in therapeutic agent delivery to hypoxic tumor regions that mirrors the $[O_2]$ dependency of radiation sensitivity that is exposing the most radiation resistant cells to the greatest cytotoxic stress. Therefore, values for K_{O_2} between 1–5 μM would be optimal; however, most current prodrug designs have K_{O_2} values $> 1 \mu M$ (1). Typically, expensive equipment not routinely available in most laboratories is required to produce $[O_2]$ levels in a suitable range to determine K_{O_2} values. The described simple and inexpensive method permits the precise control of $[O_2]$ levels within the 0–40 μM range using a simple enzymatic system. This allows the measurement of K_{O_2} values, and the acquisition of an accurate picture of prodrug activation by enzyme systems over physiological and disease relevant $[O_2]$ ranges in short term enzyme prodrug activation studies in laboratories lacking specialized equipment.

Method Development and Rationale

This $[O_2]$ control methodology is a refinement of a previous technique (6) used to generate essentially anoxic environments for cell and enzyme prodrug activation studies. Both methodologies utilize a mixture of glucose, glucose oxidase (GO) to consume O_2 and an excess of catalase (1,000 U/mL) to prevent H_2O_2 accumulation. The older method employed a nitrogen flush to initially remove the bulk of the O_2 followed by a sealed incubation to prevent further O_2 entry, and allow the enzyme based O_2 scavenging system to generate an anoxic state. The newer method is based upon establishing a steady state equilibrium between O_2 consumption and O_2 entry in a constant speed stirred solution with an air exposed surface of fixed area (Fig 1, panel A) and generates solutions with defined low levels of O_2 saturation. With appropriately chosen conditions the relationship between low (highly O_2 depleted) steady-state $[O_2]$ and the GO activity can be accurately described by a very simple mathematical relationship, $[O_2]_{ss} = k/GO$, where $[O_2]_{ss}$ equals the steady-state O_2 concentration, k is a combined constant determined experimentally for the particular apparatus/setup, and GO equals the total activity of GO in the system. This relationship was confirmed experimentally by monitoring the steady-state $[O_2]$ using a Gilson Oxygraph versus GO activity (Fig 1, panels B and C). An explanation for this simple relationship is given in the paragraph below. A detailed mathematical and experimental analysis of $[O_2]$ depletion in stirred solutions resulting from a balance between transfer across the gas phase/media interface and consumption can be found in the following references (7, 8). The Gilson Oxygraph/YSI model 5300 biological oxygen monitor used in this study has a limit of detection of $\sim 0.5 \mu M O_2$ ($\sim 0.25\%$ of the $37^\circ C$ air saturated $[O_2]$ of $212 \mu M$ (9)). However, there have been significant improvements in the sensitivity of dissolved O_2 sensors since 2009 permitting the determination of $[O_2] < 0.03 \mu M$ (10). This level of sensitivity should allow the $[O_2]$ dependency of activation of most hypoxia targeted prodrugs to be studied.

At any steady-state $[O_2]$ the rate of O_2 entry into the aqueous phase from the air equals the rate of O_2 consumption within the solution by the enzymatic system. The rate of O_2 entry will be dependent upon the following parameters; the $[O_2]$ gradient across the meniscus, the air exposed surface area, and the solution stir rate. Since we are considering hypoxic conditions where the $[O_2]$ in the solution is a small fraction of the air-saturated value, the

concentration gradient across the meniscus will be essentially constant from hypoxic to anoxic values (95–100% O₂ depletion). Additionally, since the other two parameters, exposed surface area and the rate of stir (200 rpm in our setup) are both constant, the rate of O₂ entry is also a fixed value (K_E) for all heavily O₂ depleted solutions. The rate of O₂ consumption is dependent upon the following parameters: [O₂], [glucose], and the total GO activity in the solution. Since a large excess of glucose (50 mM) is used, this value is basically unchanged throughout the experiment and can be considered a constant. The [O₂] in these studies is far below (> 10-fold) the K_m value (0.18 mM) of GO for O₂ (11) and therefore, the rate of O₂ consumption will be approximately directly proportional to the [O₂]. The rate of O₂ consumption will also be directly proportional to the total GO activity. Therefore, the rate of O₂ consumption will be proportional to [O₂] x GO activity. Under steady-state conditions the rate of O₂ entry rate equals the rate of O₂ consumption so $K_E = k[O_2]_{ss} \times \text{GO activity}$. Rearranging and combining the constants into a single value we determine experimentally for a particular setup we get $[O_2]_{ss} = k/\text{GO}$. Thus, a doubling of the total GO activity halves the steady state O₂ concentration, and a log-log plot (base 2) of the experimentally measured steady-state [O₂] versus GO activity should give a linear plot especially at low steady-state [O₂] where these approximations are more valid (Fig 1, panel C).

On addition of GO the steady-state [O₂] is usually established in < 10 minutes for experiments involving high degrees of O₂ depletion and remains stable for several hours using our apparatus and conditions (100 mM potassium phosphate buffer pH 7.4, 37 °C, 50 mM glucose, catalase 1000 u/ml, 200 rpm stir rate, ~ 0.7 cm² surface area, 2.5 mL reaction volume). The maximum steady-state duration is dependent upon the stir rate, glucose concentration and the exposed surface area to volume ratio of the reaction chamber. Study agents can be added to mixtures containing prodrug activating enzymes after the steady state is established and at various times samples can be withdrawn, quenched and analyzed by HPLC or other technique for loss of parental prodrug and the appearance of prodrug activation products. It is important to note that the sample size must be small (1–2% of the reaction volume) if several samples are to be removed sequentially from the reaction mixture. Alternatively, and favored for simplicity, a large single sample can be taken for each [O₂] and time point. That is each graphical point corresponds to a single experiment. The reason for this restriction is removing a significant sample volume raises the steady-state [O₂] because the exposed surface area, controlling O₂ entry, remains constant but the quantity of GO controlling O₂ consumption is fractionally decreased. It is possible to compensate for this GO loss preventing a change in steady-state [O₂] by adding a corrective quantity of GO in a very small volume immediately after sampling. However, if the sample size is very large (~ 0.5 mL, ~ 25% of the reaction volume), the corrective dose of GO required to maintain the steady state is slightly larger than the quantity of GO removed, because the decrease in solution depth results in an increase in the rotational speed of the liquid's surface. A simpler and more refined approach for chromophoric substrates/products would be to setup the enzyme controlled [O₂] concentration system within a cuvette. This would allow continuous monitoring of substrate/product levels without the need for sampling, for substances exhibiting useful spectral changes.

The specific reaction conditions used in all the described experiments were as follows: 100 mM potassium phosphate buffer pH 7.4, 37 °C, glucose 50 mM, GO (Sigma G7141) 0–20 units total activity, catalase (Sigma C1345) 1000 u/ml, 200 rpm stir speed, 0.7 cm² surface area, and a 2.5 mL reaction volume. The catalase and GO units used are as defined by the supplier's definition. Using the above conditions, and cytochrome p450 reductase/NADPH as the activating enzyme system, we determined a K_{O_2} value for nitrofurazone (a reductively activated antibiotic) (Fig 1, panel D). Cytochrome P450 reductase (0.01 u/ml, Sigma C8113)/NADPH (25 μ M) was chosen as the reducing system since it functions as a one electron reductant and does not significantly reduce O₂ directly (12). To maintain the NADPH pool in the reduced form these reaction mixtures also contained 1 mM glucose 6-phosphate, and glucose 6-phosphate dehydrogenase 5 u/ml. Samples were taken after 30 minutes of incubation and quenched by mixing with an equal volume of DMSO. After allowing 15 minutes for protein precipitation the samples were centrifuged and the supernatants analyzed spectroscopically. Nitrofurazone reduction was determined based upon its absorbance loss at 380 nm (86% discharged at 380 nm upon 100% reduction). The conditions were chosen to produce ~ 50% reduction of the nitrofurazone (initial concentration 50 μ M) under anoxic conditions in ~ 30 minutes. Anoxic controls contained 20 u of GO and were sealed with no head space to prevent O₂ entry. The relatively low sensitivity (~ 4 μ M O₂) of nitrofurazone's net reduction to inhibition by O₂, corresponds with the relatively low sensitivity of its cytotoxicity to O₂ inhibition reported by others (13).

Potential complications

It is important to verify that the agent being studied is not a substrate for, or an inhibitor of the glucose/GO/catalase system (examples of tests verifying this for nitrofurazone are given in the supplementary data). Inhibition, if not profound, can easily be compensated for with the use of increased levels of GO, while a small direct reduction of an agent by GO could be tolerated (and subtracted) in some types of prodrug activation experiments. If problems of the above type are encountered, an alternative substrate/oxidase couple could be tried such as alcohol oxidase from *Pichia pastoris* in combination with a methanol or ethanol substrate (14). With older less sensitive dissolved O₂ probes the use of extrapolated [O₂] concentrations may be required in some studies. In these cases the additional O₂ consumption resulting from the redox cycling activity of the prodrug/prodrug activation system could be a complicating factor resulting in the steady-state [O₂] being lower than the anticipated value precluding accurate extrapolation. In the reported nitrofurazone study, no extrapolated [O₂] values were required because the K_{O_2} value lay within our electrode's detection range. However, the use of a low level of the activating enzyme and relatively long incubation times should minimize the relative contribution of agent redox cycling to the total O₂ consuming activity. Under such conditions if a linear log-log plot of the steady-state [O₂] versus GO activity is maintained modest [O₂] extrapolation should be possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

DMSO	dimethyl sulfoxide
GO	glucose oxidase

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Fig 1.

Panel A, Illustration of the experimental setup and conditions used to generate steady-state $[O_2]$ concentrations. **Panel B**, Plot of the steady-state $[O_2]$ versus total GO (Sigma G7141) activity added to the reaction vessel in units. The values are the means of at least 3 experiments $\pm$ SE. **Panel C**, log-log plot (base 2) of the experimentally measured steady-state $[O_2]$ in μM versus the total GO units showing a linear dependency at low steady-state $[O_2]$ levels. This may permit limited the extrapolation of the GO activity required to generate lower steady-state $[O_2]$ levels if the linearity is not significantly perturbed by the presence of the prodrug/prodrug activating enzyme system. The values are the means of at least 3 experiments $\pm$ SE. **Panel D**, The determination of the K_{O_2} value for nitrofurazone. Each graphical point represents a single experiment versus an equivalent anoxic control. The smaller inserted graph is an expansion of the 0–20 μM $[O_2]$ range with an empirical curve fit based on the equation $y = (1 + Ax)^B$ ($A = 0.215$, $B = -1.11$) to match the data points. The chemical scheme illustrates the mechanism resulting in the $[O_2]$ sensitivity of nitrofurazone reduction by cytochrome P450 reductase.