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Evaluation of a self-regulated *in vitro* hypoxic system by using chemical reactions

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ABSTRACT

In this study, we established an *in vitro* hypoxic system driven by a self-regulated chemical reaction that proved effective for cell culture. The hypoxic device was modified from a 1.5 L polypropylene preservation box normally employed for food storage. Pyrogallic acid, sodium hydroxide, and sodium carbonate were dissolved in water and injected into the box. Sodium dihydrogen phosphate solution was injected into the box after 15 min. We measured the concentrations of oxygen and carbon dioxide in the box to determine viability of the hypoxic system. It maintained low levels of oxygen less than 0.2% and stabilizing levels of carbon dioxide at 5% for at least 96 h. Therefore, this device sustained a stable hypoxic environment that may be applicable for cell culture and *in vitro* studies of hypoxia.

1. Introduction

Ischemia is a very common physiological and pathological state *in vivo* [1–4]. Oxygen-glucose deprivation (OGD) is widely used as an *in vitro* model of ischemia to study the effect of ischemia on cell culture [5–7]. To perform OGD, cells are typically incubated in glucose-free media in a deoxygenated atmosphere [5,6]. Various methods have been developed to generate and maintain deoxygenated atmospheric conditions during OGD experiments [5,6,8,9]. Most commonly, gas mixtures with low oxygen content are used to obtain solutions with low levels of dissolved oxygen [8,10,11]. Consequently, special equipment is often needed to maintain these hypoxic environments, such as modular hypoxia chambers, hypoxia incubators, and hypoxic sub-chamber systems. However, these incubators tend to be large and require continual injection of oxygen-free gas to maintain hypoxic conditions within the chambers, preventing mobility of the device. In our study, we established a self-regulated hypoxic system driven by a chemical reaction.

Pyrogallic acid is used in various industrial and consumer products. It is relatively stable at ambient conditions in solution, but it rapidly reacts with oxygen when dissolved in alkaline solution. Our preliminary experiments demonstrated that an alkaline solution composed of pyrogallic acid and sodium hydroxide performed as an effective oxygen scavenger and was an ideal candidate to create and maintain a hypoxic environment *in vitro*.

pH plays an important role in the maintenance of cell culture and changes in pH can affect the physiological performance of cells in *in vitro* studies [12]. As a result, levels of carbon dioxide (CO_2) gas and pH of cell culture media (pH_m) are key parameters in cell culture maintenance. pH of cell culture media is stabilized between 7.0 and 7.4 by adding 1.2 g/L to 2.4 g/L sodium bicarbonate ($NaHCO_3$) directly into the media and pumping 5–10% CO_2 gas into the incubator, which forms a buffer system [13,14]. More specifically, ambient CO_2 gas dissolves in the culture media to produce carbonic acid (H_2CO_3), which equilibriates with $NaHCO_3$ to form a buffer pair. A buffer is an aqueous solution at a very stable pH. If an acid or base is added to a buffered solution, the pH will not change significantly or at all. Na_2HPO_4/NaH_2PO_4 and $NaHCO_3/H_2CO_3$ are common buffer pairs often used to buffer chemical reactions [15].

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The concentration of H_2CO_3 reaches equilibrium with CO_2, dependent on the partial pressure of carbon dioxide and temperature within the incubator.

Cell cultures require a stable pH condition; $NaHCO_3$ and CO_2 can maintain the pH of solutions under stable conditions [15,16]. Using these physical and chemical characteristics, we designed a simple, self-regulated *in vitro* hypoxic system that harnessed the advantages of the chemical reaction between pyrogallic acid and sodium hydroxide. Not only did the system sustain hypoxic conditions, it maintained the necessary concentration of carbon dioxide for cell culture within the box. Our design of a novel hypoxia box accounted for two key factors, the chemical solution pH (pH_s) of the reaction to maximize reaction rate of oxygen and pyrogallic acid within the box, and the CO_2 concentration to maintain stable pH of cell culture media (pH_m).

2. Materials and methods

2.1. Design of the hypoxia box

A 1.5 L polypropylene box (15 cm × 11 cm × 9.1 cm) was modified and fitted with cell culture plate brackets and solution and air valves along the wall of the box to be used as the vessel for our hypoxic system. To balance the internal and external atmospheric pressures of the box, a pressure balance bag was arranged along the inner side of the box cover and a bag valve was connected to the outside. Therefore, the box was divided into three sections: the upper section contained the airbag, the middle section provided space for cell culture plates, and the lower section was reserved for the chemical reaction. The design of the hypoxia box is illustrated in Fig. 1.

2.2. Preparation of chemical reagents

Sodium hydroxide, NaOH, (11.5 g) (96%; Aladdin, Shanghai, China) and sodium carbonate, Na_2CO_3, (2.6 g) (99%; Aladdin, Shanghai, China) were dissolved in water (55 mL). Pyrogallic acid (18 g) (99%; Aladdin, Shanghai, China) was separately dissolved in water (100 mL). Both solutions were injected into a sealed, vacuum polypropylene bag (250 mL) to allow the reagents to mix. Using a sealed, vacuum polypropylene bag prevented exposure to oxygen in the air. In addition, the reaction bag was incubated in a water bath at 25 °C to account for the release of energy from the chemical reaction. Sodium dihydrogen phosphate, NaH_2PO_4, (47.8 g) (97%, Meryer, Shanghai, China) was dissolved in water (110 mL) in a glass cup and kept in a water bath at 25 °C.

2.3. Operating the hypoxia box

Cell culture plates were placed on the brackets and the box was closed. All valves were opened, while air (120 mL) was injected into the balance bag using an injector. Next, the balance bag valve was left opened, while the other valves were closed. Sodium hydroxide and pyrogallic acid solutions were injected into the box using an infusion tube through the solution valve. The injection took 2 min to complete. During injection, the box was placed on a shaker at 48 rpm. Sodium dihydrogen phosphate solution was injected into box 15 min after the first injection, and the hypoxia box was shaken for 5 min. Finally, the hypoxia box was incubated at 37 °C.

2.4. Measuring carbon dioxide and oxygen concentrations

The concentrations of carbon dioxide and oxygen were determined using a CO_2 analyzer (XLA-BX-CO_2; Pulitong, Shenzhen, China) and an oxygen analyzer (CY−12C; Aipu, Hangzhou, China). The instruments were connected to the hypoxia box through a plastic gas channel, and a miniature air pump was added between the instruments. When the pump was turned on, air circulating through the instruments formed a gas circulation system that allowed the measurement of carbon dioxide and oxygen concentrations.

2.5. Measuring pH of cell culture media (pH_m)

Cell culture medium (2 mL), DMEM (Gibco, Rockville, MD, USA), was added to 6-well plates and placed in the hypoxia box, as shown in Fig. 1. While the box was sealed, cell culture medium was drawn out from the box using 1 mL syringe through the medium valve. pH of cell culture media (pH_m) was determined using a blood gas analyzer (i-STAT 300; Abbott, Chicago, USA).

2.6. Measuring pH of the chemical reaction solution (pH_s)

At 24 h and 96 h, the box was opened and pH of the chemical reaction solution (pH_s) was measured using a pH analyzer (PHSJ-5; INESA, Shanghai, China).

2.7. OGD testing

U87, U251, and bone marrow mesenchymal stem cells (1×10^5 cells/mL) were seeded in 96-well plates. High-glucose DMEM was replaced with glucose-free DMEM (Gibco) 2 h later, and the cells were incubated in the hypoxia box for 8 h. A control

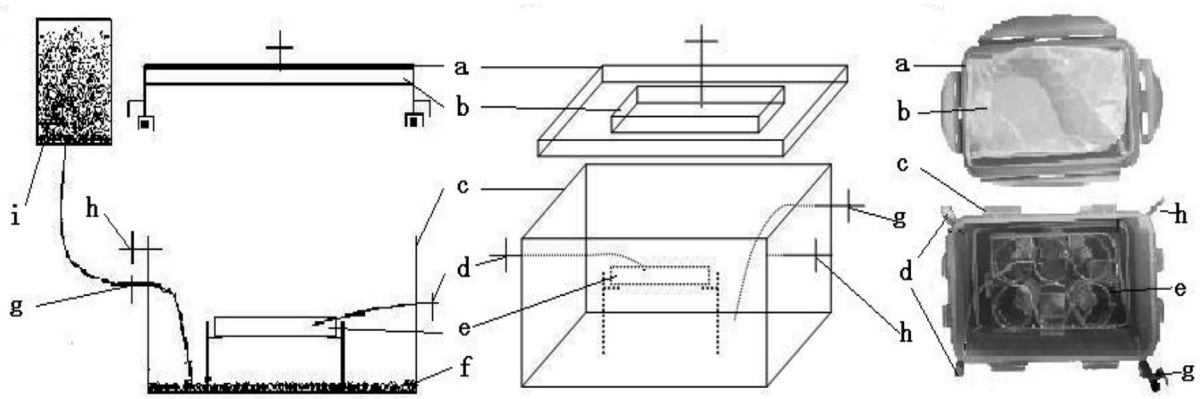

Fig. 1. Schematic of the hypoxia box. a: cover; b: pressure balance bag and valve; c: body of the box; d: cell culture medium valve; e: culture plate, f: chemical reaction solution, g: solution valve, h: air valve, i: pyrogallic acid solution.

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