On-chip multi-gas incubation for microfluidic cell cultures under hypoxia

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We developed a simple system that regulates CO_2 and O_2 levels within a microfluidic chip. This system enables long-term cell culture under hypoxic conditions without the need of a CO_2 incubator or a multi-gas incubator. Hypoxic conditions were generated using a miniature water jacket containing dissolved ascorbate as an oxygen scavenger. Formulations of the water jacket were determined that enables both 5% pCO_2 and desired pO_2 levels ranging from 5 to 15%. We also cultured PC-12 cells and primary neuronal cells from chick embryos under hypoxia and observed hypoxia-induced cell death and inhibition of neurite outgrowth. © *2014 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4902941]

Physiological oxygen concentration *in vivo* ranges from 1 to 11%[1] and is lower than the atmospheric level (20.9%). Hypoxic conditions can also result in some cellular responses such as angiogenesis,[2] cell death,[3] and differentiation.[4] To study these cellular responses, an experimental cell culture system is needed in which the oxygen concentration can be maintained at a low level. Conventional systems that create a hypoxic environment, such as multi-gas incubators and modular incubation chambers, are expensive and bulky. The cost and size of these devices are particularly problematic when one is interested in examining the degree of cellular responses under different oxygen levels. Moreover, the conventional systems do not allow researchers to mimic short-term hypoxia because they require >3 h to equilibrate the dissolved oxygen in the medium with the surrounding gas-phase O_2.[5]

To facilitate the preparation of various oxygen tensions, microfluidic oxygen gradient generators have proven to be effective in observing cellular responses under varying oxygen levels at low spatial cost.[6,7] However, to temporally and spatially stabilize the oxygen concentration gradient through gas exchange, these systems still require precise control of flow rates. As a result, most microfluidic gradient generators require cumbersome components such as syringe pumps. It is also time consuming and troublesome to calibrate the concentration gradient before each experiment. These complexities can limit research which requires specialized oxygen tensions for cell cultures.

Here, we report on a simple system for accurately maintaining the CO_2 and O_2 levels of the cell culture medium that is introduced to microfluidic channels. We designed a flat glass-bottom microfluidic chip that enables long-term maintenance of CO_2/O_2 levels in cell culture media without any incubator, recirculates media using a Braille device, and allows for observation by confocal laser microscope (Fig. 1). As shown in Figure 1(a), the microfluidic chip has two nested reservoirs partitioned with a tube made of PDMS, as previously described.[8] When the jacket (outer) reservoir has low O_2 and high CO_2 levels, the O_2/CO_2 levels of the media (inner) change likewise due to diffusion of O_2/CO_2 through the PDMS tube. The microchannel feature layer was fabricated by typical softlithographic processes with backside lithography.[9] The thin glass layer placed on top of the microfluidic channel layer can stop gas exchange at the interface of the microfluidic channels. By limiting the diffusion of gas through the channel

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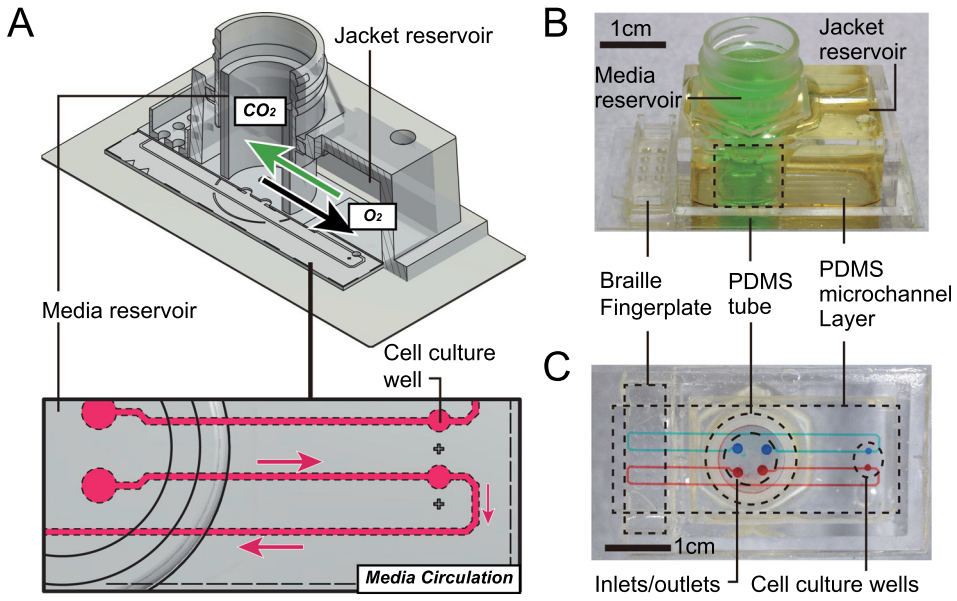

FIG. 1. Microfluidic cell culture chip with on-chip multi-gas incubation. (a) Diagram showing the maintenance of cell culture media in the microfluidic chip. Gas exchange (O_2 and CO_2) and media flow in the microfluidic channel are shown. (b) Exterior appearance of the entire cell culture chip. (c) Upper view of the microfluidic channel layer.

surface, one can accurately estimate O_2/CO_2 levels of the medium flowing through the entire length of the channels by measuring the O_2/CO_2 levels of the media reservoir.

We tested a buffer system that contains sodium ascorbate (NaHAsc) as an oxygen scavenger. Ascorbic acid ($AscH_2$) is a weak diprotic acid: it can dissociate into ascorbate monoanion ($AscH^-$) and ascorbate dianion (Asc^{2-})

$$\mathrm{AscH_2} \underset{+\mathrm{H^+}}{\overset{-\mathrm{H^+}\ pK_a=4.2}{\rightleftharpoons}} \mathrm{AscH^-} \underset{+\mathrm{H^+}}{\overset{-\mathrm{H^+}\ pK_a=11.8}{\rightleftharpoons}} \mathrm{Asc^{2-}}.$$

Although both ascorbate anions autoxidize into ascorbyl radical ($AscH^-$), deprotonation from $AscH^-$ to $AscH^-$ is unlikely to occur at near neutral pH because the midpoint reduction potential is high. Asc^{2-} easily donates electrons and forms $AscH^-$ (Ref. 10) and then forms dehydroascorbic acid (DHA). Therefore, a simplified description of the reaction is

$$\mathrm{Asc^{2-}} \xrightarrow{-e^-} \mathrm{AscH^{\bullet -}} \xrightarrow{-e^-} \mathrm{DHA}$$
$$\frac{1}{4}\mathrm{O_2(g)} + \mathrm{H^+} + e^- \rightarrow \frac{1}{2}\mathrm{H_2O}.$$

Since the second pKa of $AscH_2$ is large, the autoxidation of ascorbate is slow at near neutral pH. However, it increases by tenfold for one pH rise.[11] This slow but regulated dissociation shows how stabilization is possible in parallel with O_2 reduction and stabilization of other buffers such as bicarbonate buffer.

Temporal stability of partial O_2 pressure (pO_2) in the media reservoir was evaluated using a fiber optic oxygen sensor. Figure 2 shows the pO_2 values in the media reservoir measured for 72 h. As shown in Figure 2(a), incubating the media reservoir with NaHAsc-containing jacket solution immediately decreased the pO_2. The pO_2 then plateaued approximately after 6 h incubation. The variations of pO_2 at their plateau were within $\pm 0.3\%$ atm for all cases. The result showed that NaHAsc in the jacket reservoir effectively stabilized the pO_2 to hypoxic values for at least 3 days.

Figure 2(b) shows the relationship among the NaHAsc concentration in the jacket reservoir and the pO_2 in the media reservoir after 24 h incubation with NaHAsc-containing jacket reservoir, and the Na_2CO_3 concentration to obtain 5% atm pCO_2. As expected, we observed a

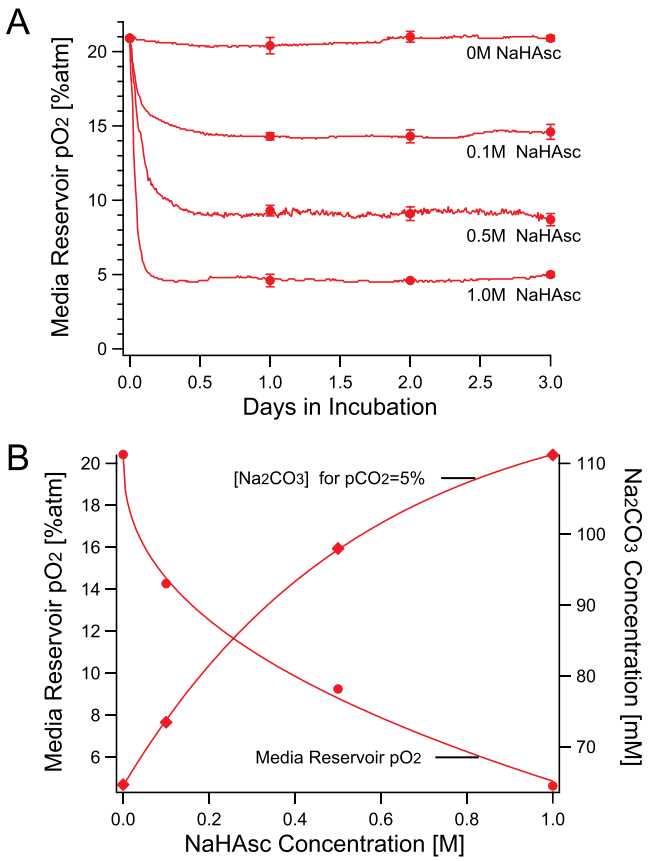

FIG. 2. Hypoxic conditioning of the microfluidic chip with NaHAsc. (a) Time evolution of partial O_2 pressure (pO_2) measured in the media reservoir in contact with jacket solutions containing 0.8 M $NaHCO_3$, 65 mM Na_2CO_3, and different concentration of NaHAsc. Observed typical pO_2 values at 10-min intervals are plotted as lines; the average values at 24, 48, and 72 h are plotted as representative values (N = 3, ±SD). (b) The relationship among the NaHAsc concentration, the media reservoir pO_2, and the Na_2CO_3 concentration when the media reservoir pCO_2 is 5% on Day 1. Interpolated Na_2CO_3 concentrations values at $pCO_2 = 5\%$ atm shown in (a) with corresponding NaHAsc concentrations were plotted.

monotonic decrease of pO_2 with increasing NaHAsc concentration. In contrast, the pCO_2 was decreased by addition of NaHAsc and could be compensated by addition of Na_2CO_3. To determine the formulation of a jacket solution from Figure 2(b), one needs to: (1) fix $NaHCO_3$ concentration to 0.8 M, (2) find a NaHAsc concentration against the desired pO_2, and (3) find the Na_2CO_3 concentration against the NaHAsc concentration obtained in (2). We can infer that the addition of ascorbate in the jacket solution changes it to the ascorbic acid molecule ($AscH_2$) and the reaction suppresses excess production of carbonic acid. As the bicarbonate buffer system reaches equilibrium and CO_3^{2-} accumulates, ascorbate donates protons to pull its equilibrium to Asc^{2-} generation, resulting in pCO_2 increase.

We examined a neuronal cell culture under hypoxia using the microfluidic and on-chip multi-gas incubation system. Figures 3(a) and 3(b) show rat adrenal pheochromocytoma (PC-12) cells cultured on-chip for 24 h under normoxia and hypoxia conditions (pO_2 approximately 5%; see Fig. 2(b)). Whereas PC-12 cells under normoxia condition (a) were healthy at 24 h, however, cells under hypoxia died (b), as shown by EthD-1 red fluorescence. These results are consistent with a report that showed hypoxia induced death of PC-12 cells,[12] in which the survival rate of PC-12 cells under 2% O_2 was 80% at 24 h and 20% at 72 h. The reason for the PC-12 cells dying more rapidly at 24 h in the microfluidic device than that of a previous report[12] may be explained by the fast diffusion velocity of gas-phase oxygen from a liquid of high surface-to-volume ratio through PDMS, a highly oxygen-permeable material compared with the velocity of conventional cell culture vessels.[5]

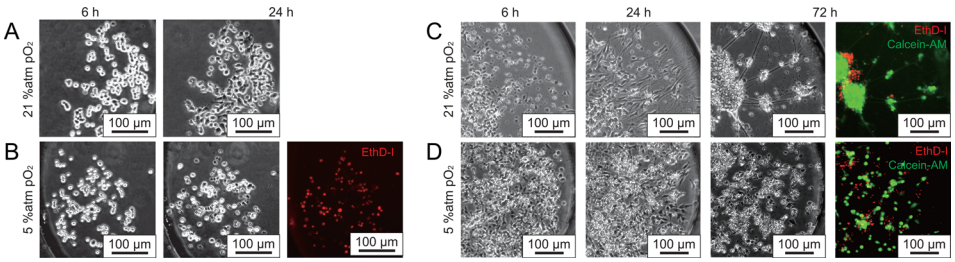

FIG. 3. Microfluidic neuronal cell cultures under hypoxia using the on-chip multi-gas incubation system. Used jacket solutions are without NaHAsc and with 1.0 M NaHAsc. The pCO_2 was adjusted to 5% atm based on data from Figure 2(b). Hours denotes the time after seeding. (a and b) Microfluidic cell culture of PC-12 cells. (c and d) On-chip culture of dorsal root ganglion neurons isolated from chick embryo.

Figures 3(c) and 3(d) show dorsal root ganglion cells from chick embryos cultured on-chip for 72 h under normoxia and hypoxia (pO_2 approximately 5%). Based on the fluorescent cell viability assay, the overall survival rate, including all cell species, was approximately 50% at 72 h. The surviving cells, however, aggregated and significantly extended their neurites only under normoxia. Neurons under hypoxia separated and extended neurites only slightly. These results reasonably agree with a previous report that showed inhibition of neurite growth and a survival rate of 75% at 48 h under 1% O_2.[13]

In conclusion, we developed a simple, standalone microfluidic chip that is suitable for on-chip cell culturing under hypoxia. Taking advantage of the slow dissociation of $AscH^-$, we successfully maintained hypoxic pO_2 levels (5% or higher) for at least 3 days. We also demonstrated successful on-chip hypoxic cell culturing of PC-12 cells and neural cells from chick embryos and found that hypoxia-induced cellular changes were similar to those observed using conventional multi-gas CO_2 incubators. The simplicity of this system is advantageous in evaluating effects of hypoxia under multiple conditions of pO_2 levels.

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