A MICROFABRICATED ELECTROCHEMICAL OXYGEN GENERATOR FOR HIGH-DENSITY CELL CULTURE ARRAYS

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ABSTRACT

We present a microfabricated electrolytic oxygen generator for use in high-density miniature cell culture arrays. The generator consists of Ti/Pt electrodes patterned at the narrow end of conical hydrophilic silicone microchannels filled with electrolyte. Surface tension forces arising from the conical microchannel geometry push generated gas away from the electrodes and down the microchannel. This bubble motion draws fresh electrolyte from an adjacent reservoir onto the electrodes. The oxygen dosage can be precisely controlled in each generator by pulse width modulation of the electrode potential. We demonstrate devices capable of continuously providing for a wide range of oxygen demands (0-200mmol/g DCW/hr) and operating for days. We also present data on corrosion conditions. We investigate pre-cure silicone additives as an alternative to plasma surface modification to obtain hydrophilic silicone surfaces. Additionally, we assay for the presence of reactive oxygen species (ROS) in aqueous medium bubbled with generated gas.

INTRODUCTION

There are an increasing number of areas where miniaturization of cell culture reactors can provide significant benefits over standard reactor or “shake flask” type incubation methods. Cell culture reactors are commonly used for optimization of some metric (such as growth rate or product yield) as a function of reactor settings (pH, oxygen, medium composition, etc.) [1]. Traditional-size cell culture reactors are large (>1L) and expensive (>\$1000); this usually limits the number of parallel experiments performed in these optimal parameter searches [2].

Specifically, the recent completion of several microbial genomes allows the development of novel antibacterial and antifungal drugs targeted to specific genes. A large cultivation parameter space must be explored to determine the function of novel genes, the optimal culture conditions for a desired bioconversion, or the most appropriate cultivation conditions for a previously unculturable microorganism. Given the large numbers of organisms that have been sequenced, unknown genes in each of those organisms, and previously unculturable organisms, a high-throughput cultivation device would allow rapid exploration of the cultivation parameter space.

Dissolved oxygen is a crucial parameter in microbial and eukaryotic cell cultures [3]. Not only do different species require vastly different oxygen conditions to thrive, but minute changes in available oxygen affect many of the metabolic pathways of the cell. Traditional methods of oxygen control usually involve bubbling oxygen or ambient gas through the cell culture suspension [4]. The specific methods vary considerably, but all make use of an external gas supply and standard plumbing and gas flow devices. Such a system would likely include pumps, valves, a gas reservoir (or an ambient air filter), and a network of fluidic channels. To control the amount of oxygen in each reactor in a high-density microarray, a complicated micro-fluidic system could be fashioned. *In-situ* electrochemical generation of oxygen in each well eliminates the need for complicated micro-fluidics by

converting a voltage (current) directly into a known rate of oxygen generation. The oxygen dosage can thus be precisely controlled independently in each well.

Silicone is gaining popularity as a MEMS material, especially for fluidic or biological applications. This is due to its elastic properties, ease of deposition, and biocompatibility[5,6]. Most commercially available silicones are hydrophobic, but in many applications this is not desirable. The use of hydrophilic block co-polymer silicones blended with a hydrophobic silicone was investigated to determine the resulting contact angles and their stability over time.

THEORY OF OPERATION

Design

The basic design schematic is shown in top view in Figure 1. A set of interdigitated electrodes lie at the narrow end of an electrolyte-filled conical hydrophilic microchannel. An appropriate potential applied between the interdigitated electrodes immersed in electrolyte generates gaseous oxygen (and hydrogen). The wide end of the channel opens into the biological medium. As a gas bubble is generated at the electrodes, it shifts towards the wider end to minimize surface tension. As the bubble travels towards the wide end of the channel, fresh electrolyte is brought onto the electrodes from an adjacent reservoir and another bubble can be formed. Un-reacted electrolyte slugs separate the bubbles in the channel and are transported into the biological suspension along with the gas. The amount of oxygen produced can be controlled by pulse width modulation of the potential. This potential is applied for a period such that the bubble is large enough to span the width of the channel, yet is only a fraction of the channel's length.

There are two mechanisms that cause the bubble to move down the channel. If the channel walls are hydrophilic and diverge from each other, then there will exist opposite but unequal forces on each end of the bubble. These forces result from the difference in channel cross section at each end of the bubble and a difference in the component of the surface tension force in the direction of the channel axis[7].

The most illustrative example is that of a channel with radial cross-section. Although the fabricated channels have rectangular cross-sections and different contact angles on different faces, a more complicated analysis obscures the basic mechanism. The surface tension at an end of the bubble can be set equal to the pressure difference across the gas/liquid interface:

$$2\pi R_1 \sigma \cos(\theta - \phi) = \Delta p \pi R_1^2 \quad (1)$$

$$2\pi R_2 \sigma \cos(\theta + \phi) = \Delta p \pi R_2^2 \quad (2)$$

Where R_1 and R_2 are the radii of the conical channel at the bubble's wider and narrower ends respectively, Δp is the pressure difference across the air/liquid interface, σ is the surface tension, ϕ is the angle between the channel wall and the channel axis, and θ is the contact angle of the channel wall. These equations can be used to find the magnitude of the forces on each of the bubble ends:

$$F_1 = 2R_1 \pi \sigma \cos(\theta - \phi) \quad (3)$$

$$F_2 = 2R_2 \pi \sigma \cos(\theta + \phi) \quad (4)$$

These forces are in opposite directions, so the total force in the direction of the wide end of the channel is:

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$$F_{net} = 2\pi\sigma[R_2 \cos(\theta + \phi) - R_1 \cos(\theta - \phi)] \quad (5)$$

Additionally, the conversion of liquid to gas at the electrolysis site results in a net volume expansion that pushes gas and electrolyte away from the electrodes. This expansion moves fluid down the channel rather than back into the reservoir because of the larger orifice size to the channel, which results in less opposing surface tension.

Hydrophilic channel walls are crucial for proper movement of bubbles from the generation site to the exit site at the end of the channel. It is well documented that silicone made hydrophilic by surface treatment with an O₂ plasma reverts to a hydrophobic state over time [8]. An alternate method of modifying the silicone is to add a few percent (by weight) of a hydrophilic silicone before curing [9]. We present data on bulk co-polymer additives that result in stable, hydrophilic silicones.

Electrolysis

Electrolytic gas generation in micro-channels has previously been investigated for various valve actuators [10,7]. The electrolysis of water with noble metal electrodes produces hydrogen and oxygen [11]:

The steady-state half-cell potentials are given in Eqs. 6, 7 ; these assume the anode environment has become acidic and the cathode environment has become basic.

Note that equal amounts of hydroxyl radicals and protons are produced. If the electrodes are spaced far from each other, this will lead to noticeable changes in solution pH. Bohm, et al used this fact to design a microtitration system [12]. Interdigitating closely-spaced electrodes ensures radical recombination and avoids undesirable pH gradients.

Several aspects of electrolyte composition must be considered. Firstly, the electrolyte employed in the bubbler must be pH-neutral and biologically compatible. As the exiting bubbles are separated by electrolyte slugs, a small amount of electrolyte enters the growth medium during bubbler operation.

Additionally, the electrolyte must be free of certain ions that may compete with the electrolysis reactions in Eqs. 6,7. The anodic evolution of chlorine, for example, has been shown to dominate the oxygen evolution reaction in saline solutions.

In the case of chlorine, although Eq. 6 is thermodynamically favored slightly over Eq. 8 at low pH, oxygen has a much higher over-potential than chlorine at current densities >100mA/cm² with conventional metal electrodes. Therefore, more chlorine than oxygen will be generated in this regime. Trace metal concentrations can also sometimes undergo transitions that can potentially compete with the oxygen reaction. We have found 1M phosphate buffer to work efficiently while producing no detectable aqueous by-products. It is therefore crucial that the complex growth medium present in the environment outside the bubbler not be allowed to enter into the microchannel as this may result in oxygen production not being the dominant reaction.

Lastly, as gas evolution is directly proportional to current flow between the electrodes, the electrolyte must be sufficiently concentrated to support the currents of interest. Assuming no competing reactions, one molecule of O₂ is generated for every 4 electrons (Eq. 6). Typical oxygen consumption rates for *E. coli* are about 40 mmol /g Dry Cell Weight/hour [3]. The total current needed to supply this demand per volume of biomass is then,

$$I = 40 \cdot 10^{-3} \frac{\text{molO}_2}{\text{gDCW} \cdot \text{hr}} \cdot \rho \cdot N_A \cdot \frac{4e^-}{O_2} \cdot \frac{q_e}{e^-} = 4.3 \frac{\mu A}{\mu L} \quad (9)$$

where $\rho=1$ g/l is a typical biomass density [3], $N_A=6.023 \cdot 10^{23}$ molecules/mole is Avogadro's number, $q_e=1.609 \cdot 10^{-19}$ C is the electron charge. As discussion of bubble column gas transfer is beyond the scope of this paper, we will assume the generated gas is fully utilized by the biomass above the bubbler chip [4].

Given the well-known oxygen overpotential at metal electrodes and the above current requirement, it is often necessary to operate at potentials in excess of the thermodynamic half-cell potentials for the anode and cathode reactions. As we increase the potential drop across the electrodes, other reactions come into play at the electrode surfaces. Of particular interest are reactions that generate reactive species with aqueous lifetimes greater than a few seconds as these could be potentially anti-microbial if exhausted into the biomass.

We can calculate the rate of electrolyte consumption from the desired oxygen production rate. The electrolyte reservoir must then be large enough to supply electrolyte for the maximum operational lifetime.

$$V_{consumed} = 80 \frac{\text{molO}_2}{\text{hr} \cdot \mu L} \cdot V \cdot \frac{2H_2O}{O_2} \cdot \frac{18g}{\text{mol}} \cdot \rho_w = 288 \frac{\text{nl}}{\text{hr}} \quad (10)$$

where ρ_w is the density of water and $V=200\mu L$ is the volume of the biomass.

Corrosion

To control oxygen generation, the operating potential is pulsed at frequencies varying from 0.1 – 2.0 Hz. Direct application of these pulse trains forces the electrodes to continuously alternate polarization during operation. Alternating current polarization has been shown to lead to corrosion and roughening of noble metal electrodes [13]. Specifically, those noble metals which readily dissolve hydrogen, such as platinum and palladium, exhibit rapid corrosion rates. Hoare showed that hydrogen-dissolving electrodes absorb evolved hydrogen when negatively polarized and expel hydrogen when positively polarized [13]. The resulting movement of the hydrogen atoms resulted in break-up of the electrode surface, leading to roughening and eventual degradation.

Corrosion of noble metal electrodes can be avoided by alternating between a fixed operating potential and an unbiased, or 'floating' condition during operation. This can be readily accomplished by applying the desired pulse train via a relay. During the applied pulse, the electrodes see a fixed potential; on the pulse's falling edge, the relay opens and the electrodes are allowed to float. This eliminates the alternating polarization and thus the motive force for the hydrogen absorption while maintaining the same actuation signal.

MATERIALS AND METHODS

Fabrication

The devices are fabricated on 4" silicon wafers (Fig. 2). A silicon nitride deposition (6000 Å) is followed by an n-type polysilicon deposition (1.5μm, 10 Ω/□). The polysilicon is patterned into traces and passivated with a second silicon nitride deposition. 5x5μm vias are cut into the nitride and Ti (500 Å) and Pt (2000 Å) layers are sputtered and patterned over the vias into 10x10μm electrodes using a lift-off process. Resist (SJR 5740) is spun 50μm thick and patterned to form a mold for channels. Wafers are then dipped in Sylgard Prime Coat and allowed to dry for 4 hours. Room temperature vulcanizing (RTV) silicone (Dow-Corning 734) is spun onto the wafer (150μm thick) and cured at room temperature. The wafer is then subjected to UV light to expose the buried resist. The wafer is diced into 1cm x 1cm chips. A 2mm x 1cm trench is cut in the center of the chip; this trench is

perpendicular to the channels thus providing the fluidic connection between the channel and the biological suspension. The photoresist is removed by immersion in developer with ultrasonic agitation for 60min. For devices requiring surface modification, the chip is exposed to a 50W O₂ plasma at 600mT (Technics PEII-A); this yields hydrophilic silicone surfaces. The completed chip can then be filled by capillary action when immersed in electrolyte for a few seconds. Each chip contains 13 identical electrolytic gas generators.

Contact angle experimental setup

Silicone samples for contact angle measurements were prepared by dipping pre-cleaned glass slides (Fisherbrand 12-550A, 25 x 75 x 1mm) into 50 mL test tubes filled with uncured silicone. Additives were hand-mixed into the silicone before dipping the slides. The samples were degassed in a vacuum chamber for 20 min. and allowed to dry while suspended from one end. This method generated samples with silicone on all surfaces of the glass slide; a small (<1mm) edge bead formed at the bottom end of the slide while drying. Dow-Corning 734 (RTV) silicone was used. Additives used in this paper are GE Silicones SF1488 (40-60 cstks), Gelest DBE-712 (20 cstks, 600MW) and Gelest DBE-224 (400 cstks, ~10,000MW); all are dimethylsiloxane-ethylene oxide block copolymers. A Kruss G10 goniometer with computer video capture and a KSV Sigma701 tensiometer were used for contact angle measurements. Goniometer contact angles were determined by curve fitting on a captured image using a circle-fitting algorithm provided by the software.

Reactive Oxygen Species (ROS) Quantification

Soft plastic centrifuge tubes (United Scientific Products, 1.5 mL flattop, MCT-150) were roughly cut at the narrow end to leave a ~2mm hole. The tubes were then cleaned in isopropanol and blown dry. The large mouth of the tubes were bonded to completed bubbler chips using RTV silicone (Dow-Corning 734) and allowed to cure overnight. The tubes were then filled with 1.5 mL phosphate buffer (1M). An indigo trisulfonate colorimetric assay (modified from Standard Method 4500-O₃B [14]) was used to quantify the steady-state ROS concentration in the buffer as follows. Indigo trisulfonate discolors in the presence of reactive oxygen species. During each experiment, the 13 bubblers on the chip were operated simultaneously to generate gas into a filled tube. After 1 hour of operation, 1.2 mL of phosphate buffer was quickly removed and added to 0.3 mL of indigo trisulfonate solution II [14]. Optical densities were measured at a wavelength of 600nm with a path length of 1cm; all measurements were taken with DI water as a blank (O.D.₆₀₀=0). A series of controls were made with 1.2 mL of virgin phosphate buffer and 0.3 mL of indigo trisulfonate; mean and standard deviation between controls was calculated with measurements from 10 control samples. ROS concentrations were calculated from O.D.₆₀₀ as per [14].

In a separate experiment, the 13 bubblers on the chip were operated simultaneously to generate gas into a tube filled with pH indicator dyes to visualize pH gradients (either 10:1 H₂O:Alizarin Red S [pH 5-8] or 10:1 H₂O:Bromophenol Blue [pH 3-5]). pH measurements were also made in a Horiba Twin pH meter following recommended calibration procedures over the course of 2 hours.

RESULTS AND DISCUSSION

Figure 3a-d shows the bubbler under normal operation. A minimum delay of 0.5s between potential pulses was found sufficient to allow the bubble time to exit the electrode area. With a 3.3V, 0.5s on, 0.5s off pulse, each bubbler has a maximum oxygen generation rate of 30nL/s. Shorter off-time results in

pulses being applied while there is still gas over some electrodes. Additionally, as bubbles lose velocity near the mouth of the channel, smaller delays between pulses leads to gas coalescence and significantly larger exiting bubbles. In the extreme of a constant potential being applied, the generated gas coalesces completely and soon fills the entire channel, including the electrode area; gas generation drops significantly as only a few electrodes are covered with electrolyte.

Exiting bubble size was strongly dependent on silicone contact angle. In channels with hydrophobic contact angles, exiting bubbles remained trapped at the channel mouth. Bubbled gas then inflated the trapped bubble until the buoyancy force overcame surface tension (usually resulting in bubbles with diameters >2mm). A delay of more than 12 hours between oxygen plasma modification and operation resulted in devices which exhibited this behaviour. This motivated our search for time-stable hydrophilic silicones.

Corrosion

As discussed above, application of an AC potential directly to the electrodes results in rapid corrosion of the electrodes. Figure 4 shows the effect of corrosion on electrode surface. Figure 5 shows bubbler lifetime degradation. As Ti/Pt electrodes disintegrate, the amount of current through the bubbler drops. Actuation through a relay results in no appreciable corrosion; bubblers have been operated continuously for up to 24 hours with no detectable drop in current (data not shown). Operational lifetimes are currently limited by the size of our reservoir.

Reactive oxygen radicals (ROS)

Presence of reactive oxygen species was measured using an indigo trisulfonate colorimetric assay (Methods, above). Table 1 shows the results of representative experiments. At our operating potentials, the O.D.₆₀₀ variation between controls was greater than that of the sample; this limits the measurement resolution of our current set-up to ~100 µg/L (100ppb). This is mainly due to our small sample size; additionally, we are restricted to small (1cm) optical path lengths for the optical measurement. Ozone concentrations >10 µg/L (10ppb) have been shown to affect *E. coli* metabolism [15,16,17]. *E. coli* suspension cultures grow successfully on gas generated by our chips in both rich and minimal medium (data not shown).

Table 1

Optical Density at 600nm wavelength (O.D. ₆₀₀)			
Controls (std. dev)	Controls (mean)	4.0V 60 min.	5.5V 60 min.
0.013	0.419	0.442	0.384

No pH changes were detected in pH measurement and indicator dye experiments (DI water, nominal pH 6.9±0.2).

Silicone Contact Angles

Silicone surfaces are usually made hydrophilic by oxygen plasma or acid surface modification [18,19]. It is well documented that surface modified silicone rapidly loses its hydrophilic nature (usually ~1-2 days) [8,20]. Recently, blends of polydimethylsiloxane (PDMS) silicone and amphipathic siloxane copolymers (such as polyethylene oxide) have been suggested as a way of obtaining hydrophilic silicones [9]. In an effort to develop time-stable hydrophilic silicones, an RTV silicone was mixed with different concentrations of dimethylsiloxane-ethylene oxide block copolymer and allowed to cure. Contact angles were then measured with a goniometer. Interestingly, we noted that water drops initially showed a hydrophobic profile but relaxed over ~5sec into a stable hydrophilic profile. Table 2 shows blends used and initial contact angles; goniometer data taken over 3 weeks indicated contact angles were stable (data not shown).

Table 2

Blend (% by weight)	Goniometer Contact Angle (quasi-static)
3% DBE-712	35°
6% DBE-712	20°
1% SF1488	45°
3% SF1488	70°
3% DBE-224	55°

Tensiometer data was then taken on identical samples in order to shed light on the observed dynamic aspects of the contact angle. Modified silicone samples were lowered and raised into 150 mL of DI water at 10mm/min over a 10mm section of sample to obtain advancing and receding contact angles. After 10 successive dips, the surface tension of the liquid was measured with a platinum standard plate (provided with the tensiometer) at 20mm/min (Figure 6) and the water was replaced with fresh DI water. The tensiometer data suggests the block copolymer additive leeches into the water over time, changing its surface tension and thus the measured contact angle. Additionally, the surface tension of 15 mL of water extracted from the bottom of the tensiometer beaker was essentially that of pure DI water (data not shown); this further indicates that the leached co-polymer is sequestered almost exclusively at the air-water interface. Figure 7 shows tensiometer contact angle data over 30 days for bulk modified silicone as well as O₂ plasma surface modified silicone; no aging effect other than that attributed to copolymer leeching was observed. Since the copolymer leeching appears to be a diffusion process, this data suggests microchannels could be patterned whose surface properties change strongly as a function of fluid velocity.

Electrolyte interface

In order to test the separation of electrolyte from the biological suspension, 10µm polystyrene beads were placed in the destination solution and their motion filmed. In the course of 30 minutes of observation, 4 beads were able to enter the channel. These beads were able to travel up the channel a distance approximately equal to the length a bubble occupies in the channel. None of the beads reached the electrolysis site as the bubbles were much shorter than the channel. Each of these beads got caught in the air/water interface of a bubble and this prevented them from moving further down the channel. The trapped beads were then pushed towards the channel end via the movement of the interface. The caught beads were not cleanly ejected from the channel, but instead their position oscillated along the channel axis as they were released from one interface only to be pulled back into the channel and caught on another interface. All 4 of the beads that made it into the channel eventually broke out of the oscillation and were ejected from the channel within minutes.

The motion of the bubbles resulted in the movement of nearby beads into a formation. (Fig. 8) As bubbles exited the channel, they pushed the nearby beads in the solution away from the channel orifice. Due to the low Reynolds number at this length scale, beads that did not come in direct contact with the bubble, but were within ~3 bubble diameters of the channel end, could be seen moving in response to a bubble exiting the channel. The bubble briefly remained attached to the end of the channel until the buoyancy force was greater than the surface tension force holding it down. When the bubble broke free, water moved in to fill the space, and the surrounding beads were pulled in towards the channel end. The repetition of this process resulted in an arc-shaped pile of beads forming at a distance of 1 bubble diameter from the channel orifice center.

CONCLUSION

We successfully microfabricated and tested an electrolytic oxygen generator for use in high-density miniature cell culture arrays. A single generator chip can support oxygen consumption

rates >100mmol/DCW/hr and operate for lifetimes in excess of a day. Additionally, the copolymer silicones investigated suggest interesting properties applicable to a broad class of microfluidic devices.

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Figure 1: Schematic top view of bubbler.

Figure 2: Process cross-section after removal of photoresist.

Figure 3: Bubbler in operation. a) Bubble is formed over electrodes. b-c) Bubble travels down conical microchannel. d) Bubble is exhausted into medium (becoming spherical). A voltage pulse train of the right frequency generates a continuous bubble train.

Figure 4: Electrode Corrosion due to AC excitation in electrolyte. A 0-5V, 1 Hz square wave was applied for 1 hour, 1M phosphate buffer, pH = 7.2 was used as the electrolyte. a) Excitation through relay (no corrosion). b) Excitation without a relay (corroded anode). The lithographic misalignment between the metal and via layers on this wafer did not appear to have any effect on bubbler operation.

Figure 5: Effect of corrosion on oxygen generator lifetime. A 0-5V, 1 Hz square wave was applied for 1 hour; 1M phosphate buffer, pH = 7.2 was used as the electrolyte. a) Excitation through relay (no corrosion). b) Operation without relay results in gradual corrosion of Ti/Pt electrode; this both reduces operating lifetime and introduces bacteriocidal Ti/Pt contaminants into exit stream..

Figure 6: (right) Surface tension of water vs. number of silicone sample immersions into water. 10mm of sample were immersed at 10mm/min into 150mL DI water. Surface tension of pure water is 71 mN/m at STP.

Figure 7a,b: (below) Contact angle aging data for modified silicones. Measurements were taken every 2 days for 27 days. 10mm of sample were immersed at 10mm/min into 150mL DI water. Data presented for 1% SF1488 / 734 blend.

Figure 8: Channels isolate electrodes/electrolyte from foreign particles in surrounding medium. Chips pre-filled with 1M phosphate buffer were immersed in water containing 10 μ m beads. Any beads that entered the channel were trapped by the gas-liquid interface formed by exiting bubbles; additionally, the liquid motion caused by the exiting bubble aggregated nearby beads at a distance approximately equal to the exiting bubble diameter.

a) Density of unperturbed beads on substrate surface. b) Bead arrangement at channel mouth, t=10min. c) Bead density at channel mouth, t=30min.