GAS PERFUSABLE MICROFABRICATED MEMBRANES FOR HIGH-DENSITY CELL CULTURE

Colin A. Cook[1], Yang Liu[1], Jianming Lu[2], Nanhai Chen[2], Yuman Fong[2], and Yu-Chong Tai[1]
[1]California Institute of Technology, Pasadena, CA, USA
[2]City of Hope, Duarte, CA, USA

ABSTRACT

We report for the first time the design, fabrication, and feasibility of a high-density cell culture system capable of a cell substrate density of 40 cm^2/cm^3, greater than 250× current commercially available systems. This design provides oxygen directly from gas perfusable membranes on which the cells are grown to overcome oxygen delivery limitations in traditional culture systems. With translation in mind, the membranes are composed only of medical grade PDMS and Parylene to mitigate contaminant leaching concerns.

INTRODUCTION

Oncolytic virotherapies [1], although having shown immense promise to treat cancers, are currently limited by the cost of producing sufficient doses of virus from adherent mammalian cells for trials and eventual clinical use. Since the major expense of virus production is the GMP facility space for cell culture [2], it is informative to use the metric "cell substrate surface area / system volume" to compare the effectiveness of current systems: roller bottle towers (0.04 cm^2/cm^3), cell factories (0.05 cm^2/cm^3), wave bioreactors (0.16 cm^2/cm^3). Analysis of mass transport in these systems reveals that the low density of current production methods arises from oxygen transport limitations due to its low solubility in media [3]. By splitting oxygen supply and soluble nutrient supply using separate gas and liquid compartments it is possible to overcome these limitations to achieve greater cell densities as demonstrated in experimental oxygen perfused dialysis cartridge type bioreactor systems (3.2 cm^2/cm^3) [4]. Recognizing the art is still well below the theoretical ceiling of 500 cm^2/cm^3 for adherent cells, we sought to apply MEMS approaches to achieve the next node of high-density cell culture.

We conceptualized a hollow, gas perfusable membrane system in which cells could grow directly on the membrane, receiving their oxygen from permeation through the membrane and soluble nutrients from the media in which the membrane was submerged. To maximize the surface area to volume ratio, the membranes would be stacked producing an alternating structure of gas, membrane, media, membrane, gas, and so forth (Figure 1). The media compartment of the layers would be open along the edges to allow for gentle perfusion of media and to facilitate cell seeding onto and subsequent harvesting off of the membranes.

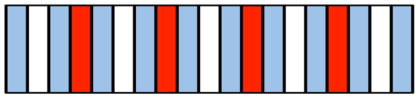

Figure 1: Conceptual structure of stacked, gas-perfusable membranes. Membranes (blue), media compartment (red), gas compartment (white).

MATHEMATICAL MODELING

Oxygen Delivery

We considered the growth of adherent mammalian cells on a gas perfusable membrane that could directly supply cells with oxygen. PDMS was selected as the structural component of the membrane due to its oxygen permeability and Parylene HT was selected as a coating layer, having previously been shown to facilitate cell adhesion [unpublished data] and having superior oxygen permeability compared to other Parylenes. Mechanical and fabrication considerations established the thickness of the PDMS layer of the membranes to be 120µm. Parylene thickness was informed by analytical solutions of oxygen transport across the membrane to cells in the media compartment. A thickness of 1µm was found to maintain oxygen tension within 90% of the perfusing gas (21% O_2, 5% CO_2, 74% N_2), ensuring adequate oxygen supply (Figure 2).

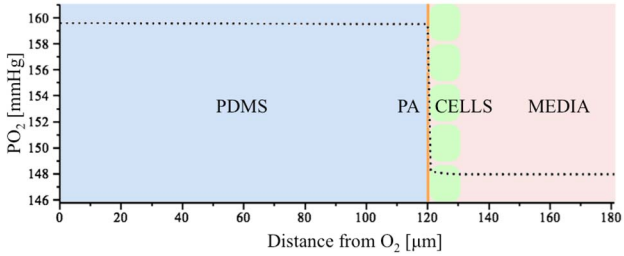

Figure 2: Oxygen tension throughout one surface of the gas perfusable membrane of PDMS coated with 1µm PA-HT and the corresponding cell/media compartment.

Shear Forces

Most adherent cell lines are highly sensitive to shear forces [5], above which they fail to grow (e.g. critical shear rates: FS-4 cells = $3s^{-1}$, CEF cells = $7s^{-1}$). In microcarrier systems that can theoretically achieve very high surface area to volume, shear forces induced by the mixing necessary for oxygen delivery limit maximum cell density far before the cells would run out of substrate area. We therefore modeled the necessary perfusion rate through the media compartment needed to prevent metabolite limitation. In Vero cells, a commonly used cell in vaccine production, glutamine has been identified as the most limiting nutrient after oxygen [6].

Firstly, the maximum perfusion velocity, U, is limited by shear, α, experienced by the cells within the media compartment of height H:

$$U_{max} = \frac{H \cdot \alpha_{crit}}{6} \quad (1)$$

Mass balance of nutrient content within media perfused through the compartment yields the differential equation:

$$\frac{\partial C}{\partial t} = -U \cdot \frac{\partial C}{\partial x} - \frac{2 \cdot V_{max}}{H} \quad (2)$$

This can be solved to find the nutrient concentration along

the flow path, where C_0 is the initial concentration of the nutrient, V_{max} is the maximum consumption rate:

$$C(x) = C_0 - \frac{2 \cdot V_{max}}{U \cdot H} x \quad (3)$$

We enforce the condition that the nutrient concentration be maintained 4× above the Michaelis constant concentration, K_m, at which cell growth/consumption is half-maximal, so that cell growth is at least 80% of maximum everywhere. This gives the maximum length of the membrane:

$$L_{crit} = \frac{(C_0 - 4 \cdot K_m)(U \cdot H)}{2 \cdot V_{max}} \quad (4)$$

Finally, we plug in for the maximum perfusion velocity:

$$L_{crit} = \frac{(C_0 - 4 \cdot K_m) \cdot \alpha_{crit} \cdot H^2}{12 \cdot V_{max}} \quad (5)$$

Utilizing parameters (Table 1) for Vero cells [3], [6], plots of critical membrane length versus membrane spacing were produced for oxygen (assuming no membrane permeation), glutamine, and glucose (Figure 3).

Table 1: Cell parameters for simulations

Metabolite	C_0	$V_{max}\left[\frac{amol}{cell \cdot s}\right]$	K_m
Oxygen	56μM	56	8.5μM
Glutamine	2mM	31	30μM
Glucose	150mM	86	4mM
$\alpha_{crit} = 1\ s^{-1}$		$\rho = 5 \times 10^5\ \frac{cells}{cm^2}$	

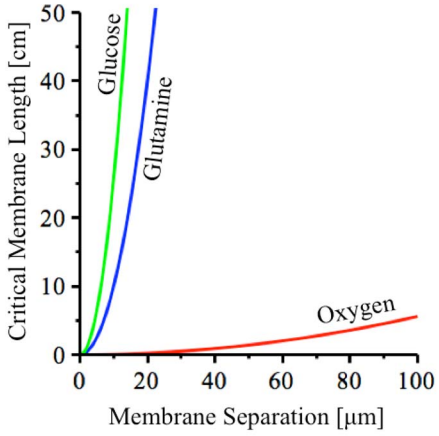

Figure 3: Critical membrane length versus membrane separation for different limiting metabolites. Oxygen is observed to be more than 100× limiting compared to glutamine, justifying the need for delivery via membrane permeation.

DEVICE DESIGN AND FABRICATION

Microfabrication

We fabricated stackable hollow membranes out of PDMS (for its oxygen permeability and resilience) that could be perfused with gas to oxygenate the adjacent media-filled compartments through permeation. The membranes have one flat face and one face with spacing posts (200μm diameter; 500μm pitch) so that when stacked there is a sufficient gap for cell entry and growth (120μm). Similar posts are contained within the membranes to tie the two halves together under pressure-driven gas perfusion. The membranes were cast in microfabricated dry-film photoresist molds (10cm × 10cm × 240μm) and the two halves were glued together to form a single hollow membrane (480μm thick), Figure 4A. To facilitate cell attachment we coated the membranes with 1μm of Parylene HT and subsequently treated with oxygen and ammonia plasma (200mT O_2, 1 min, 50W; 200mT NH_3, 1 min, 50W), previously shown to enable cell attachment.

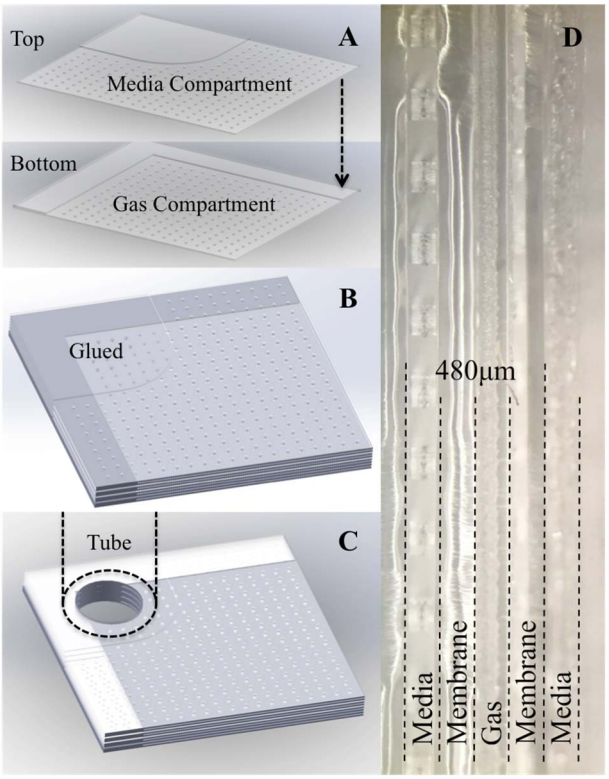

Figure 4: Fabrication of stacked membranes. A) Top and bottom halves of PDMS membranes glued together along border then coated with 1μm Parylene HT. B) Membranes are stacked creating alternating gas and cell/media compartments; corners are glued. C) Diagonal corners are punched and silicone tubing is glued to connect all membranes to a common gas source. D) Cross sectional image of stacked fabricated membranes with overlays indicating media and gas compartments.

Permeability Testing

The oxygen permeability of the fabricated membranes was determined by monitoring the change in oxygen concentration in a sealed, stirred container of 900mL DI-water that housed a 100cm² membrane perfused with 100% oxygen at 1atm. From Henry's law and membrane permeation theory, it is known for this system that $\frac{dP_{O_2\%}(t)}{dt} = \frac{\sigma \cdot 2A \cdot [100\% - P_{O_2\%}(t)]}{V \cdot H}$. By extracting $\frac{dP_{O_2\%}(t)}{dt}$ (Figure 5A), the oxygen permeability of the membrane can be calculated: $\sigma = 1.7 \times 10^{-18}\ \frac{mol\ O_2}{\mu m^2 \cdot s \cdot atm}$. This is about 6× lower than predicted by a simple bilayer model of a Parylene HT on PDMS membrane and likely occurs due to penetration of Parylene HT into the PDMS, rather than a purely surface coating.

Additionally, the uniformity of oxygen delivery across the membrane was probed utilizing an oxygen sensitive solution that turns from clear to pink in the presence of oxygen (1M NaOH, 0.133M glucose, 13µM resazurin). A membrane was submerged in the solution and perfused with oxygen. A uniform pink aura was observed across the surface of the membrane indicating that the flux of oxygen was even (Figure 5B).

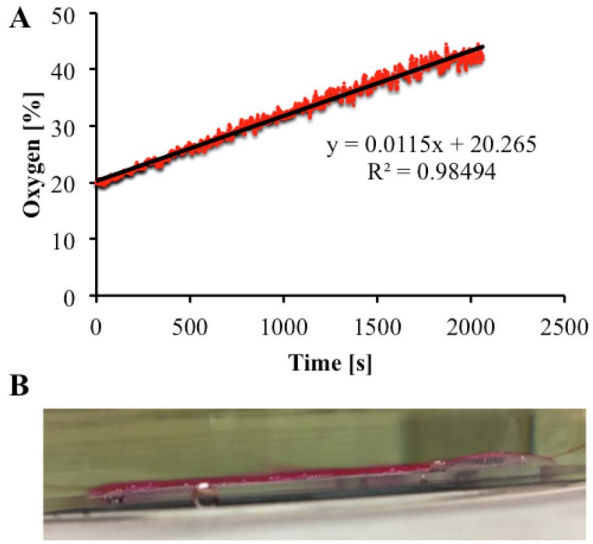

Figure 5: Oxygen delivery from membranes. A) Oxygen permeability of membranes measured by monitoring change in oxygen tension in a sealed, mixed container of water over time. B) Resazurin solution visualizes oxygen permeation through perfused membrane into solution, indicated by the clear to pink transition of the dye.

CELL EXPERIMENTS

Independent Oxygen Supply

In conventional cell culture, oxygen supply is through diffusion of oxygen from atmosphere into the media. However, in a multi-stacked culture system internal membranes must be able to independently supply sufficient oxygen to cells. We sought to demonstrate the capacity for the membranes to act as an independent oxygen supply by growing CV-1 cells on membranes in an anoxic atmosphere. Following seeding (2×10^4 cells/cm^2; media: DMEM (Corning #10-017-CM) with 10 % FBS (Omega #FB-12)) for 24hrs on membranes under normal culture conditions (21% O_2, 5% CO_2, 74% N_2), the membranes were moved into an anoxic atmosphere (5% CO_2, 95% N_2). One set of membranes was perfused with (21% O_2, 5% CO_2, 74% N_2) while the other was not perfused with gas. After 24hrs the cells were observed and those on perfused membranes were healthy and growing while those without perfusion had balled up and were dying (Figure 6).

Therefore, the delivery of oxygen through the membranes is sufficient to maintain cell viability without the need for media convection or diffusion from atmosphere.

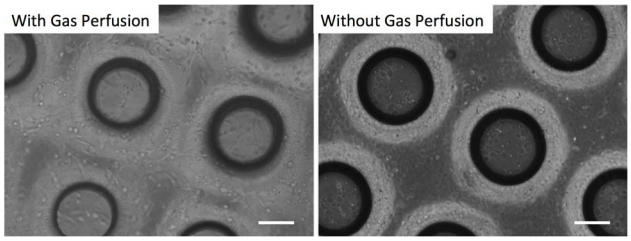

Figure 6: Oxygen delivery from membranes can solely maintain cell survival. CV-1 cells seeded on membranes for 24hr then transferred to anoxic environment with (left) or without (right) gas perfusion for 24hrs. Scale = 100µm.

Bioreactor Design

A bioreactor was designed to test the feasibility of culturing cells on stacks of gas perfusable membranes (Figure 7). An imaging-compatible enclosure was designed to house the membranes and allow for simultaneous perfusion of the cell compartment with media using a peristaltic pump and perfusion of the gas compartment using a regulator and flow meter (21% O_2, 5% CO_2, 74% N_2). The system was maintained in a cell incubator at 37°C. Sterilization of the bioreactor was done via perfusion with ethanol, then sterile water, and subsequently cell media through the media compartments. External ports were fitted with 0.22µm filters.

The individual membranes were joined into a single gas filled compartment by stacking and subsequently gluing diagonal corners with PDMS, punching though the stack, and connecting to silicone tubing (Figure 4B,C). The system was leak tested to avoid bubble formation in the media during gas perfusion. The setup provides a surface area to volume ratio of 40 cm^2/cm^3.

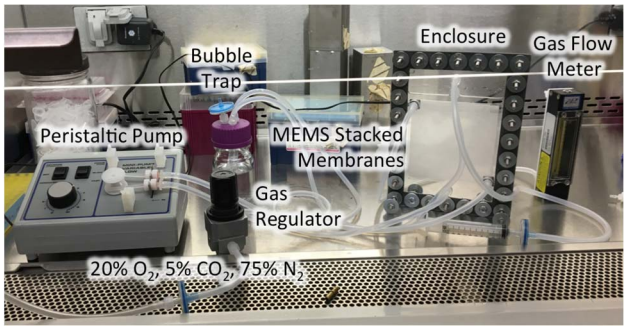

Figure 7: Experimental setup and bioreactor. Gas perfusable membranes (stack of 4) are placed in imaging-compatible enclosure with hermetic feed-throughs allowing for gas and media perfusion. A peristaltic pump controls media perfusion through the cell/media compartment. A gas regulator and flow meter control gas perfusion. An inline bubble trap maintains atmospheric pressure and bubble-free media perfusion.

Multistack Cell Seeding

Cells can be dispersed evenly throughout the membranes by injection into the media perfusion loop followed by a short period of circulation (1-min) for mixing. In order to achieve uniform seeding of both sides of the membranes, the bioreactor was placed in a horizontal orientation and flipped every 30min. Cells began anchoring within 3hrs of seeding and within 20hrs of seeding had begun to spread (Figure 8).

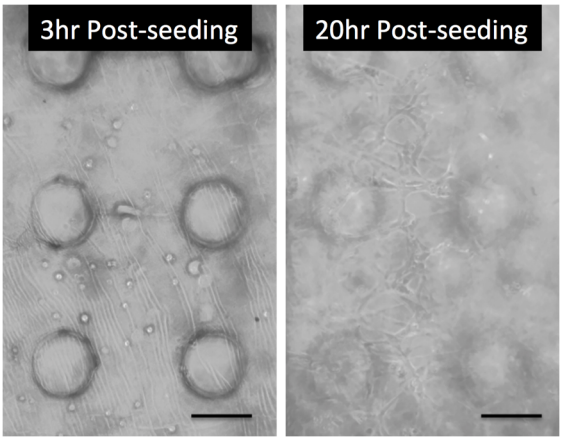

Figure 8: Cells can be seeded and attach on stacked membranes. (Left) CV-1 cells were seeded throughout 4-stacked membranes by injection into the media perfusion loop and were adherent by 3hrs. (Right) Cells began spreading within 20hrs. Scale=200μm.

Multistack Cell Expansion

Finally, the culture of cells in a multistack format was demonstrated in the bioreactor containing four membranes. MDBK cells were seeded at an initial density of 2×10^4 cells/cm^2 and cultured for 4 days. The membranes were subsequently imaged individually using a light microscope to validate the growth of cells on each of the layers (Figure 9). Relatively uniform and high-density cell growth was observed across each of the membranes.

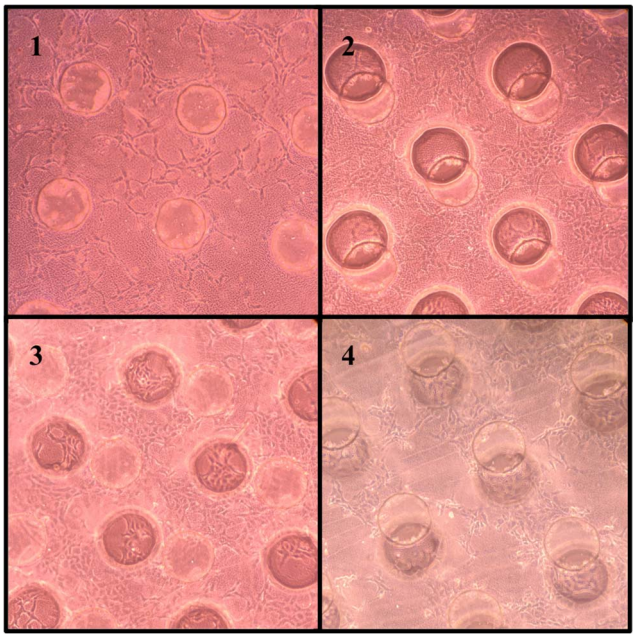

Figure 9: Images of individual membranes from stack of four at 10x magnification on Day 4 of MDBK cell culture. Image panels depict 1.3mm×1.3mm areas.

CONCLUSIONS

Gas perfusable membranes provide a significant surface area to volume ratio that can support high-density growth of adherent cell cultures. By delivering O_2 and exchanging CO_2 directly from the cell growth substrate we have overcome the oxygen limitations of conventional cell culture systems. The next most limiting metabolites for cell growth are comparatively soluble in water when compared to the rate at which cells consume them (e.g. glutamine, serine). This enables very low perfusion rates through the media compartments, which in turn allows for closely spaced membranes without exceeding critical shear limits on the cells. The approach we have demonstrated can be scaled in a straightforward manner by adding additional membranes in the stack or increasing their size. Additional work is being done to reduce the thickness of the gas perfusable membranes, enabling even higher cell densities to be achieved. We believe this approach to high-density cell culture holds significant promise to reduce oncolytic virotherapy vaccine production costs. Future studies will look at the ability of cultured cells in the system to produce virus.

REFERENCES

[1] S. J. Russell, K.-W. Peng, and J. C. Bell, "Oncolytic virotherapy," *Nat. Biotechnol.*, vol. 30, no. 7, pp. 658–670, Jul. 2012.

[2] L. J. Ausubel, M. Meseck, I. Derecho, P. Lopez, C. Knoblauch, R. McMahon, J. Anderson, N. Dunphy, V. Quezada, R. Khan, P. Huang, W. Dang, M. Luo, D. Hsu, S. L. C. Woo, and L. Couture, "Current good manufacturing practice production of an oncolytic recombinant vesicular stomatitis viral vector for cancer treatment.," *Hum. Gene Ther.*, vol. 22, no. 4, pp. 489–97, Apr. 2011.

[3] A. R. Oller, C. W. Buser, M. A. Tyo, and W. G. Thilly, "Growth of mammalian cells at high oxygen concentrations.," *J. Cell Sci.*, vol. 94 (Pt 1), pp. 43–49, 1989.

[4] K. Ku, M. J. Kuo, J. Delente, B. S. Wildi, and J. Feder, "Development of a hollow-fiber system for large-scale culture of mammalian cells," *Biotechnol. Bioeng.*, vol. 23, no. 1, pp. 79–95, Jan. 1981.

[5] M. S. Croughan, J.-F. Hamel, and D. I. C. Wang, "Hydrodynamic effects on animal cells grown in microcarrier cultures," *Biotechnol. Bioeng.*, vol. 29, no. 1, pp. 130–141, Jan. 1987.

[6] S. Quesney, A. Marc, C. Gerdil, C. Gimenez, J. Marvel, Y. Richard, and B. Meignier, "Kinetics and metabolic specificities of Vero cells in bioreactor cultures with serum-free medium.," *Cytotechnology*, vol. 42, no. 1, pp. 1–11, May 2003.

ACKNOWLEDGEMENTS

Thanks to the Caltech MEMS Lab members for their guidance with techniques and fabrication. In memory of Lillian Boasie and Gweneth Goodman.

CONTACT

*C.A. Cook, tel: +1-443-8542070; ccook@caltech.edu.com