A MICRO LUNG CHIP TO ASSESS AIR POLLUTANT EFFECTS

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ABSTRACT

This paper reports the fabrication and initial testing results of a dynamic micro lung chip to first time monitor the air pollutant effects on the lung epithelial cells. On the micro lung chip, it was demonstrated to culture lung epithelial cells (A549) on an inserted porous membrane; to real-time monitor the cell growth over time by measuring Trans-Endothelial-Electrical-Resistance (TEER) values with integrated micro electrodes; and to allow preliminary evaluation of cell responses and tracer permeation to the exposure to an air pollutant. After 24-hour exposure of the lung cells to an air pollutant (Na_2SO_3), the fabricated chip produced higher permeability by +908%. It also showed a lower TEER by 27% under the exposure to a simulant of 100 µM FITC-4k dextrans.

INTRODUCTION

Air pollution imposes an increasing concern on health as well as environment. For example, an air pollutant, Particulate Matter with a diameter of less than 2.5 µm, $PM_{2.5}$, exposed 92% of the world population beyond the safety guideline concentration of 10 µg/m^3 in 2014 [1]. Its exposure concentration even reached to a high concentration of 43 µg/m^3 $PM_{2.5}$ in most cities in 2014, according to the report of World Health Organization (WHO) [1].

Air pollutants have been suspected to be a main cause for lung cancers, which was estimated mostly by statistical methods that computed an average value from a large number of cases and thus fail to describe individual cases. Previous literature reported that the exposure of 10 µg/m^3 PM_{10} and 5 µg/m^3 $PM_{2.5}$ resulted in higher incidents of lung cancers by 22% and 18%, respectively, among 2,095 lung cancer cases in 14 European cities [2]. Another statistical study reported that the exposure of 10 µg/m^3 $PM_{2.5}$ and NO_2 caused higher association with lung cancers by 11% and 6%, respectively [3]. Such a statistical methodology, performed over a large number of >1,000's sample cases, inevitably ignored individual differences in exposure levels, periods or environments.

To overcome such a limitation, several *in vitro* lung models have been developed and utilized evaluating air pollutant effects on the cultured lung cells of individuals [4,5]. These *in vitro* models incorporated a culture place for lung cells that could be exposed to air pollutants, which provided specific, repeatable and low-cost alternative to conventional statistical or in-vivo models. Such evaluation results showed that a certain degree of causal correlation exists between air pollutants and cell viability. For example, higher expression of chemo-attractant protein of interleukin-8 (IL-8), a precursor for potential cancer development, was obviously expressed under an application of 0.1 ppm ozone and 0.1 to 10 mM of Na_2SO_3 in a well-plate *in vitro* lung model. Such an expression even exhibited autonomously increasing relationship by 5 times under increasing concentrations of the Na_2SO_3 from 0.1 to 10 mM.

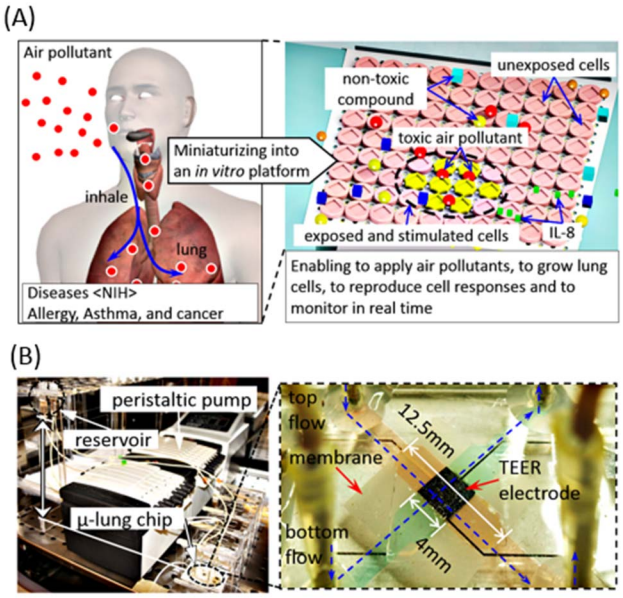

Figure 1: (A) Concept of a micro lung chip. Inhalation conveys an air pollutant to the lung and causes cells response. The micro lung chip mimics such as process in a controlled platform with integrated electrodes. (B) Photos of the testing platform (left) and a fabricated device (right): Top and bottom channels formed a cross-section spot (4×4 mm^2) for cell culture. At the cross-section, a thin porous membrane was placed and served as a cell culture site.

Previous *in vitro* lung models can be grouped into two categories: static and dynamic models, depending on the existence of flows of air pollutants to the cultured cells. Static models utilized one-time injection of air pollutants into a sealed container to cause an exposure to lung cells, such as in conventional well-plate or transwell models [11]. However, they failed to represent a physiological fluid flow that are known to be critical in forming in vivo-like environments, such as mechanotransduction effects [6]. Dynamic models [7-10] incorporated micro airways to mimic air flows, representing closer to real exposure. Some models successfully reported toxicity results of naphthalene and its metabolites [7] and utilized two-phase flow of air and media [8]. However, dynamic models provided only end-data points, lacking the real-time monitoring capability that is critical in understanding transient variations.

We report the development of a dynamic micro lung chip that is capable of providing real-time monitoring and such results for the first time. Specifically, we report fabrication, culture of lung epithelial cells and integration of sensors of the micro lung chip.

METHODS

Structure & Fabrication

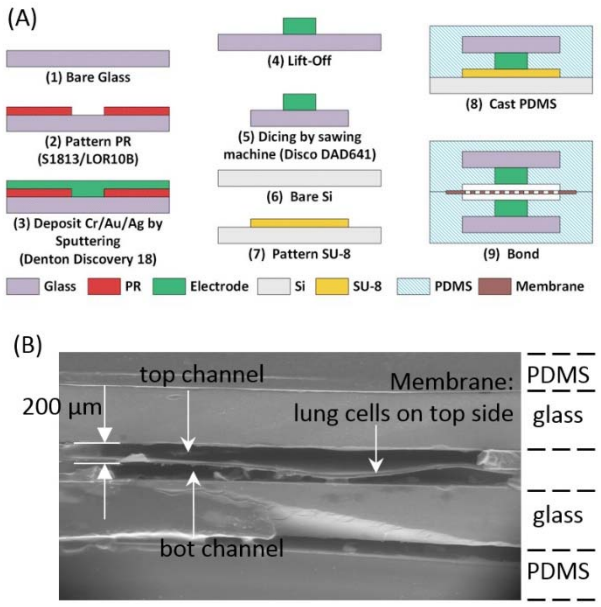

Figure 2: (A) Fabrication process flow of the micro lung chip. (B) SEM image of the cross section of the micro lung chip. It consists of two parallel channels, one porous membrane, and two electrical glass chips.

The micro lung chip was fabricated by bonding one sheet of porous polycarbonate membrane and two PDMS channels containing trans-endothelial electrical resistance (TEER) electrodes (Fig.2(A)), as reported in previous papers [12-14]. The TEER electrode was fabricated by patterning photoresist and sputtering 20/150/800 nm Cr/Au/Ag layer on a glass wafer. The patterned glass wafer was then diced to a single TEER chip with a footprint of 18×25 mm^2. Separately, to construct the cross-passing channels, conventional poly-dimethylsiloxane (PDMS) molding technology was utilized. First, a SU-8 2075 photoresist layer was patterned to form a 200 µm-height mold on a silicon substrate. Then, the SU-8 mold was covered with a PDMS layer (10:1 elastomer:curing agent) and cured at 65℃ for 6 hours. The cured PDMS was cut into a piece of 35 ×25 mm^2 and was punched to form holes for media flow in both the top and bottom channels. When a pair of PDMS layers were bonded, it contained an inserted porous membrane of polycarbonate to form a cell culture area. To assemble the top and bottom channels and the porous membrane, the PDMS layers were bonded using spin-coated and stamped 1:1 ratio PDMS pre-polymer toluene, as previously described in [15]. Then, fluidic connection were attached at inlets and outlets of each channel.

Cell culture

Human alveolar epithelial cells (A549) were cultured on the polycarbonate culture spot in Dulbecco's modified Eagle's medium: Ham's F12 supplemented with 10% fetal bovine serum (Corning, NY), 1% amphotericin B (Cellgro, VA), and 1% penicillin/streptomycin (Lonza, GA). First, the cell line was pre-cultured in a T75 flask over 5 to 6 days until it reached 80% confluency. The culture conditions included a temperature of 37℃ and a humidified atmosphere of containing 5% CO_2 in an incubator (NU-4750, Nuaire). The media were exchanged every 3 to 4 days. Second, the pre-cultured cells were induced into the microchip. To assist the induced cells to adhere onto the polycarbonate membrane, adhesion layers of fibronectin and collagen IV (both 100 µg/ml) were previously coated on the top side of the polycarbonate membrane. Then adhered lung cells (A549) were seeded at the concentration of 6.0×10^4 cells/cm^2 by flowing cell suspension and cells were culture at no-flow overnight. Then, the lung epithelial cells were then cultured for 4 to 5 days under media flow provided by a peristaltic pump, and the culture media were changed daily. For a comparison purpose, the same cell lines were also seeded in static transwell models (Transwell 3412, Corning, NY) at the same concentration of 6.0×10^4 cells/cm^2 as the micro lung chip.

TEER measure and testing

After the cell seeding, the cell growth was monitored by real-time measuring TEER values (EVOM2 epithelial voltmohmeter, WPI) every day from day 0. TEER values were determined in Ω·cm^2 according to the equation (1),

$$TEER = (R - R_{blank}) \times A \quad (1)$$

where R was the total resistance (Ω), R_{blank} was the background resistance (Ω) on day 0, and A was the cell culture area (cm^2). The TEER measurement utilized a constant 10 µA AC current at 12.5 Hz while measuring the resistance. The area of the TEER electrodes was designed to be the same as the cell culture area (4×4 mm^2) to ensure the uniform distribution of ion flow across lung epithelial cells.

When TEER values were saturated, three different media of 1 mM Na_2SO_3 (Sigma, MO), 40 ng/ml epidermal growth factor (EGF, Sigma, MO), and carrier buffer (Hank's Balanced Salt Solution, Cellgro, NY) were applied to the chip for 24 hours. During the air pollutant application and permeability measurement, TEER values were measured every 6 hours to monitor the electrical signals, which indicated the cell responses. The TEER values were monitored

After the 24-hour application of the different solutions, permeability was measured at each conditions with 100 µM FITC-4k dextran at 1 mm/s velocity corresponding to *in vivo* conditions [16] and at no-flow in the transwell. The amount of permeated compound collected from the bottom channel was then measured using a spectrometer (495/525 nm, SpectraMax Plus 384 Microplate Reader). The permeability coefficients P were calculated using equation (2) [17],

$$P = \frac{J_s}{AC_L} \quad (2)$$

where P is the permeability coefficient (cm/s), J_s is solute flux across the cell layers (mol/s), A is the membrane area (mm^2), and C_L is concentration (mol/m^3) on the top channel.

Cell images and interleukin-8 production

After the permeability test, the fluorescence images of cells were monitored with a microscope (EVOS FL Auto, Thermo Fisher) by staining nuclei with DAPI (Enzo, NY) in order to confirm cell growth and formation.

After 24 hours of incubation with Na_2SO_3, EGF, and buffer, the culture supernatants in the top channel of the micro lung chip and in the apical chamber of the transwell

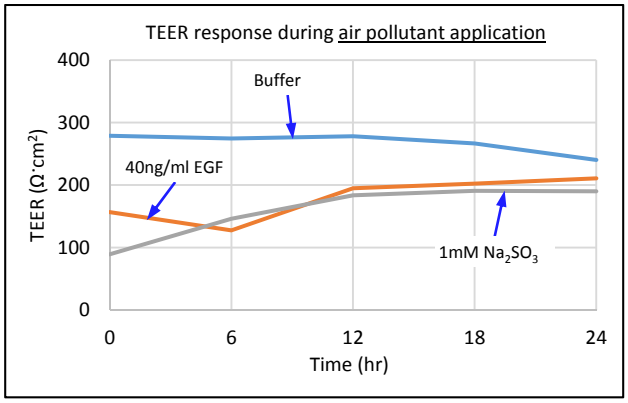

Figure 3: Evaluation of air pollutant Na_2SO_3 by TEER measurement.

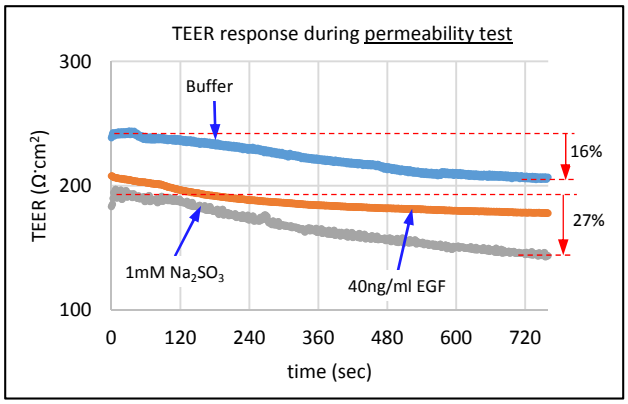

Figure 4: Evaluation of cell responses by TEER measurement during FITC 4k dextran application.

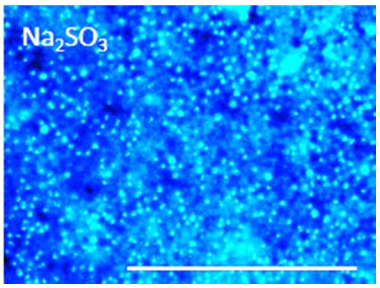

Figure 5: Fluorescence images of the lung epithelial cells (A549) with DAPI staining under the exposure to Na_2SO_3, for 24 hours. Scale bar is 400 μm (10x scale).

were collected, and the concentration of IL-8 in the supernatants was quantified by using the human IL-8 ELISA kit (Thermo Fisher) according to the manufacturer's instructions.

RESULTS & DISCUSSION

The micro lung chip was successfully fabricated into a single chip (Fig. 2(B)) and established by culturing A549 lung epithelial cells in the chip under continuous media flow (Fig. 5). The micro lung chip generated cell responses in terms of TEER value changes by +113%, +34%, and -14% after 24 hours of application of Na_2SO_3, EGF, and carrier buffer, respectively (Fig. 3). The TEER values were monitored during cell permeability test as well. The final TEER measurement showed some decrease in their values by -27%, -15%, and -16% under the exposure to the Na_2SO_3, EGF, and carrier buffer, respectively (Fig. 4). The TEER measurements proved a feasibility to real-time predict cell responses at each proposed conditions.

Cell image of Na_2SO_3 application stained by DAPI reagent indicated that cells were successfully seeded and

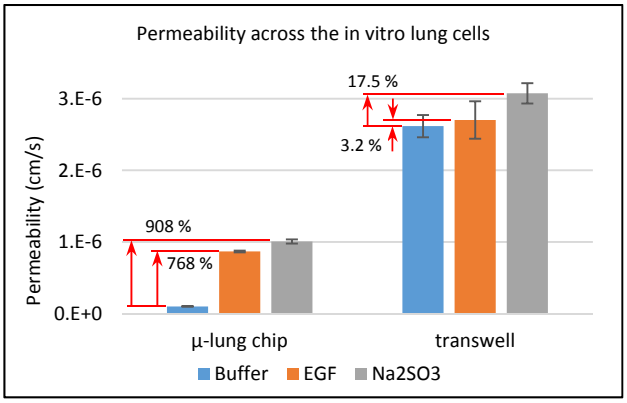

Figure 6: Permeability evaluation at the micro lung chip and transwell with the buffer, 40 ng/ml EGF, and 1mM Na_2SO_3 by applying 100 μM FITC-4k dextran.

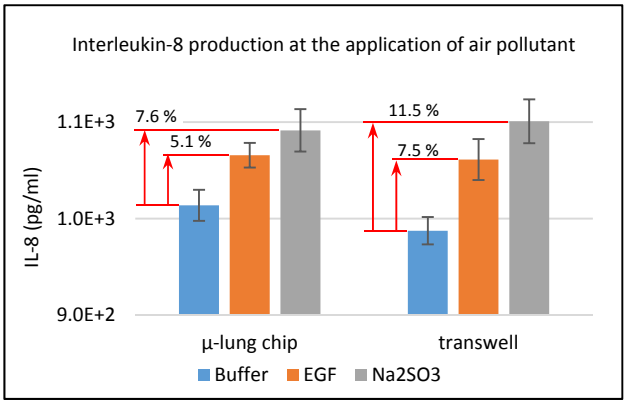

Figure 7: Measurement of Interleukin-8 production. The produced interleukin-8 in the culture supernatants of the micro lung chip and transwell plate was measured by human IL-8 ELISA kit.

cultured alive (bright sites) across the culture area in the micro lung chip (Fig.5). The cell image confirmed the permeability test was performed with the confluent and well-characterized lung cells.

Measurement results also showed that the exposure of an air pollutant produced higher permeabilities of compounds across the epithelial cells by 908% than control (buffer exposure), as shown in Fig. 6. The permeabilities were measured as 1.01×10^{-6} and 8.67×10^{-7} cm/s under the exposure to Na_2SO_3 and EGF, respectively, which is significantly higher than 9.99×10^{-8} cm/s to buffer media. Such increased permeation under the exposure to Na_2SO_3 and EGF implied loosening of tight junctions among cells under the exposure to air pollutants and higher possibilities of compound transportation being enabled through endocytotic pathways.

For comparison purposes, permeability was also measured with a conventional static *in vitro* model, transwell. Providing a relevant fluid flow enabled to predict permeability of FITC-4k dextran representing drug and air pollutant closer to an in vivo expectation than no-flow. The static model measurement also showed a similar trend: 17.5% higher permeability for the exposure to the air pollutants than buffer. Note that the measured permeability at our dynamic platform was 3 times less than conventional static model, implying a closer correlation to in-vivo.

Measurement results also showed that cancer-related protein expression of interleukin-8 increased under the

exposure to air pollutants as shown in Fig. 7. The IL-8 expression under the exposure to EGF and Na_2SO_3 was measured as 1.07×10^3 and 1.09×10^3 pg/ml, which was higher than under the exposure to buffer media (1.01×10^3 pg/ml) by 5.1 and 7.6% higher, respectively (Fig.7). The IL-8 expression at the transwell plate showed a similar trend: enhanced expression values under the exposure to Na_2SO_3. The expression values were measured as 1.06×10^3 and 1.10×10^3 pg/ml, each representing enhancement by 7.5% and 11.5% in comparison to the control.

CONCLUSION

This study reported the development of a dynamic micro lung chip comprised with micro PDMS channels, porous membrane, and glass electrode chips. The human alveolar epithelial cells were effectively cultured in the established model under a continuous flow of 0.15 mm/s. Finally, the air pollutant effects on the cultured lung cells were converted into electrical signals of TEER measurements. Then the affected cells were again evaluated by a compound permeation with the application of FITC-4k dextran application at a velocity of 1.0 mm/s and the permeability test showed a higher permeability under the exposure to Na_2SO_3 than buffer. Furthermore, the interleukin-8 production in the supernatants collected from the top channel of the micro lung chip was assessed by ELISA. The IL-8 measurement confirmed higher IL-8 production of the cultured lung cells under the exposure to Na_2SO_3.

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