CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Chemicals and instruments used in this study are indicated in the appendix.

2.2 Methods

2.2.1 Establishment of direct alveolar epithelial cells-air exposure prototype.

The direct alveolar epithelial cells- air exposure prototype (exposure chamber) contained two main parts, chamber control zone and culture zone. At the chamber control zone, tested air was aspirated into the chamber at a flow rate comparable to human breathing (5 liters/minute). The air passed through a UV light zone. The UV light could destroy any bacteria in the air. The air was then passed through an impactor which could select only particles less than 2.5 micrometers in diameter to pass into the culture zone. The culture zone was an incubator controlling the temperature at 37^{o}C and 95% humidity. Human epithelial lung cells (A549) were cultured by the air/liquid interface technique and were directly exposed to the air. The air was then aspirated out of the chamber.

In detail, an exposure chamber contained five important parts.

1. Air input unit
2. UV sterility system
3. Impactor
4. Exposure chamber

5. Temperature and Humidity controller

Air input unit

Air input unit was the part for corrected air test side into the chamber. This unit is designed to a minimum power loss by based on the principle of fluid dynamics. The shape of the entrance affects to the energy loss of air which flow to inside the pipes system. This research designed the channel, as shown in Figure 2.1 along with installed the curtain wall to block insect or other contaminants do not want to clog up the system.

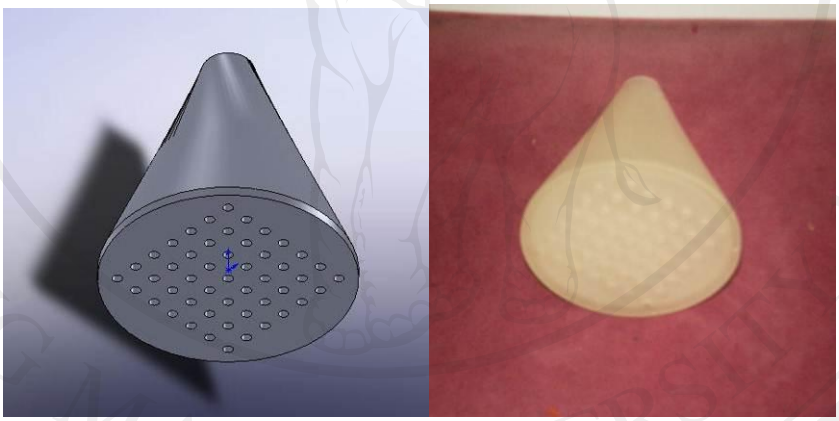
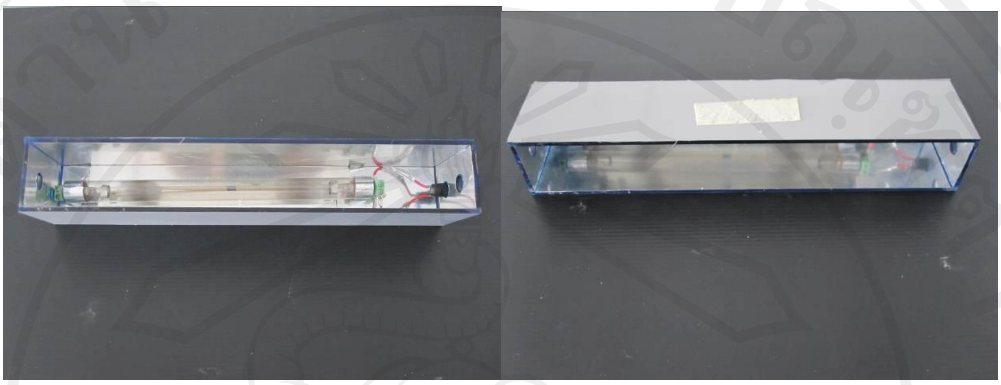


Figure 2.1 Air input unit with curtain wall

UV sterility system

The air passed through a UV light zone. The UV light could destroy any bacteria in the air by exposure to UV-C at wavelength between 100-280 nm. We used UV-C light bulb radiated at wavelength 253.7 nm put in a sterile box with reflect plates. The air passed through would be disinfected before passing into the particulate

filter system. UV lamp was installed at the bottom of the box as shown in Figure 2.2 (a).



(a) (b)

Figure 2.2 The UV lamp installed in a box for sterilization

Impactor

Cascade impactors are widely used to classify particle sizes for industrial hygiene purposes and for environmental and toxicological studies. However, to obtain accurate size distributions, the cut of characteristics of each impactor stage must be determined, as the cut of characteristics are often quite different from the designed specifications. Among the various ways of calibrating an impactor, e.g., gravimetric, fluorometric, counting and electrical techniques, in this study we chose to use the gravimetric and counting methods, and the fluorometric method was used to evaluate wall losses. The gravimetric method was used to determine the collection efficiency by weighing the mass of particles collected on the substrate. The calibration process is time-consuming and hard to apply to the calibration of nanoparticle size range because of the sensitivity of the microbalance and the length of the collection time.

The counting method allows the collection efficiency to be determined by counting the number of particles upstream and downstream of the impactor stage.

To control the flow rate of air which need to be comparable to human respiration rate (5 liters per minute), an impactor air filtration system was designed. This impactor would allow the particle at desirable size (PM2.5 or PM10) to pass through the chamber.

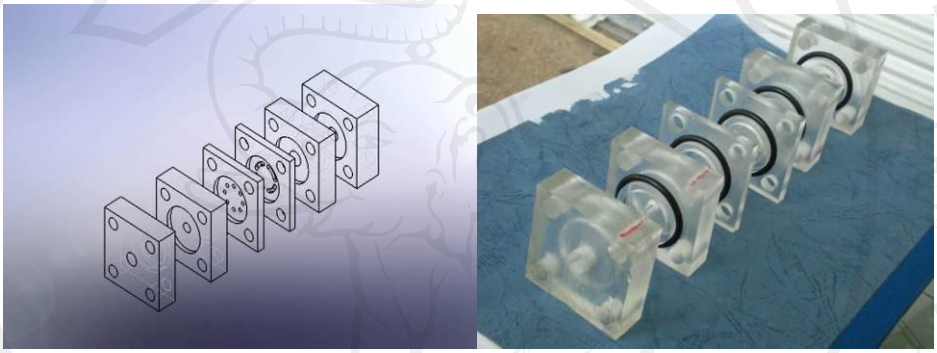


Figure 2.3 Three-dimensional images of air impactor filter and prototype

The impactor was designed base on the Stokes's theory when particle passes through the nozzle and is directed towards the impaction surface (substrate), it changes its direction abruptly to form bends in the streamlines. Particles with aerodynamic diameters larger than a certain value (called the cut-off size of the impactor) have enough inertia to cross the streamlines and impact onto the substrate. The smaller particles which have insufficient inertia follow the streamlines and remain suspended in the air and are not collected. The primary parameter which governs the collection of particles in an impactor is the Stoke's number (Stk) which is a dimensionless parameter that can be used to predict whether a particle will impact on an impaction plate of a stage or will follow the air streamlines out of the impaction

region and remain air-borne. This impactor was designed to choose separation between PM10 and PM2.5.

Impactor system was composed of two parts. First part can filter particles less than 10 microns and second part can filter particles less than 2.5 micron. The structure of impactor to filter particle size less than 10 or 2.5 micron are shown in figure 2.4 and 2.5, respectively.

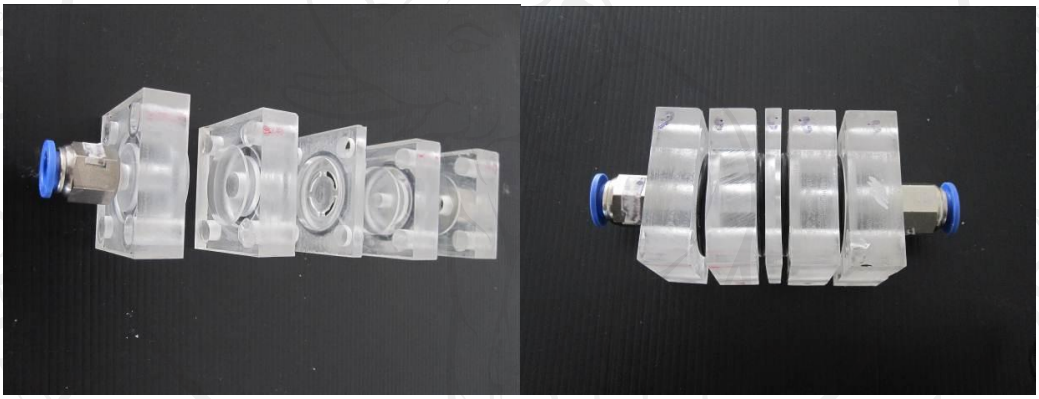


Figure 2.4 Construction of the impactor for separation of PM10

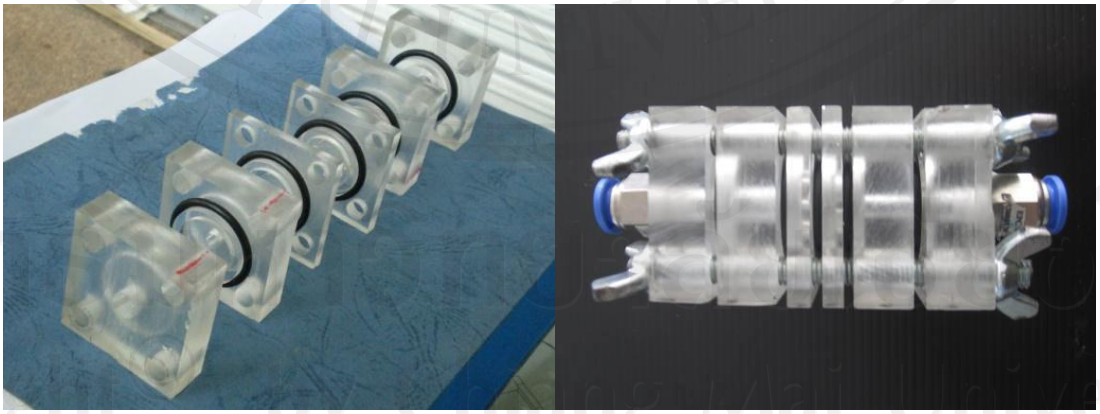


Figure 2.5 Construction of the impactor for separation of PM2.5

Construction of Exposure chamber

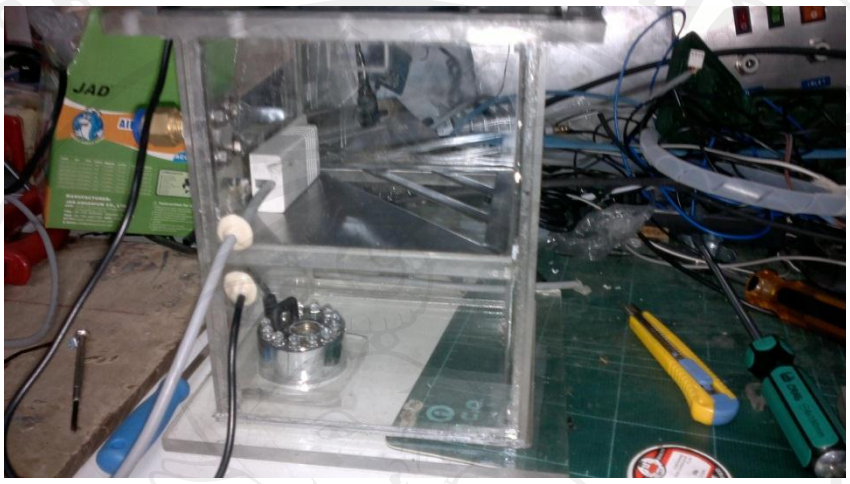


Figure 2.6 The exposure chamber consists of three parts; temperature and humidity sensors, heater and moisture system.

1. Temperature and humidity sensors

The SHT15 digital sensor was used in this chamber. This sensor can measure the temperature in the range of -40 to 120 ◦C. It can also measure humidity in the range of 0 to 99% with a resolution is 1%.

Figure 2.7 The SHT15 digital sensor

2. The heater system

We used the resistors to generate the heat for chamber. This resistor is wrapped by coils and is coated outside by ceramics. We used 5 series of 10 W resistors to generate the heat.

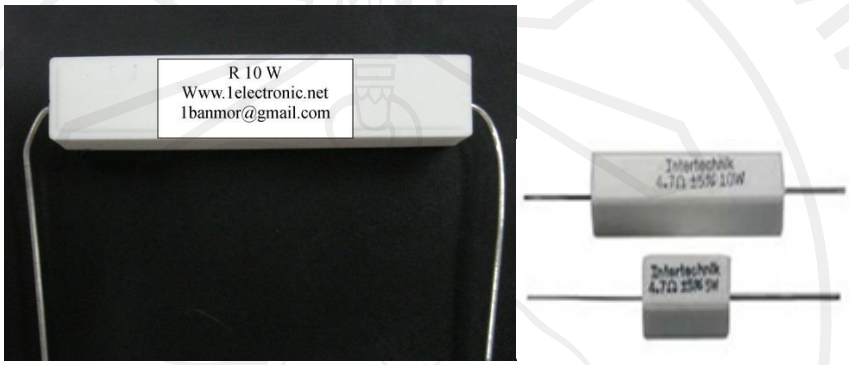


Figure 2.8 Ten Watts resistor

3. Moisture system

To produce moisture in the camber, a fog machine or an ultrasonic humidifier was used. Humidity can be generated up to 99% RH. Ultrasonic humidifier can generate the fog by using high frequency waves to change water molecule into a very small droplets. This water droplet would make chamber humidified.

Figure 2.9 The Ultrasonic Humidifier

Temperature and humidity controller

In this study, the temperature and humidity was controlled by the AP-105. The AP-105 was an index to measure temperature and humidity with High-resolution digital sensors. It can measure Temperature in the atmosphere at range from -40 to 120 ◦C, the resolution was 0.1 degrees and It can measure the humidity from 0 to 99% RH the resolution was 1% (10 to 90% accuracy) and can remember data Min, Max, of temperature and humidity and could reset values on the machine can be set up 5 relay Control, (1 is on the board of the need to expand) the temperature or humidity can be controlled by each Relay. It controlled hot or cool temperature by set High and Low Temperature or humidity. It can set the Timer for turn on/off Relay in the second unit. Beside, we controlled cold temperature depend humidity for control the fan or pump. In addition, there is also a port for communication. RS232 (RS485) for send data to the PC with a Network PC and easy to use with only two buttons on the instrument for measure the temperature and humidity variations, as shown in Figure 2.10

Figure 2.10 Temperature and Humidity controller

In this study, the culture zone was an incubator controlling the temperature to temperature 37 ◦C and humidity at 95% RH, which is similar to the temperature and humidity of the human lung. The temperature and humidity were controlled by SHT15 sensor as shown in figure 2.11 and 2.12.

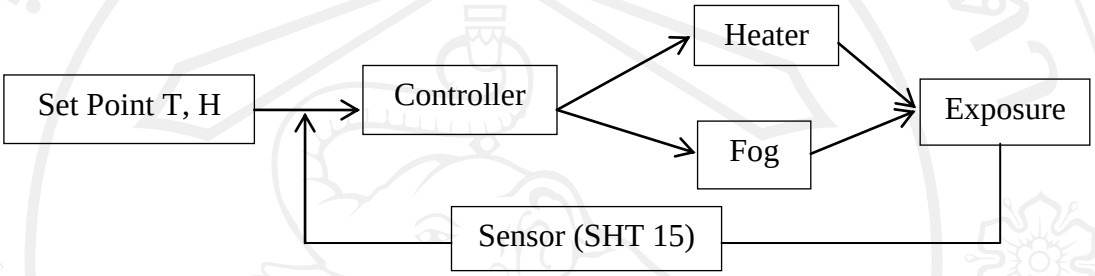


Figure 2.11 Schematic diagram of the control system of temperature and humidity in chamber

Figure 2.12 Temperature and humidity controller of exposure chamber

2.2.2 Cell culture

Human pulmonary type II-like epithelial cells (A549) were cultured in DMEM supplemented with 10% fetal calf serum and 1% v/v antibiotic containing 10,000 IU/ml penicillin, 10,000 IU/ml streptomycin and 250 µg/ml amphotericin B. Cultured cell were incubated at 37 °C in a humidified 5% CO_2 incubator.

For conventional cell culture, newly confluent cell layers were enzymatically removed by using trypsin/EDTA (Gibco, USA), and resuspended in the culture medium. Cell viability was measured by 0.4 % (w/v) trypan blue (Sigma, USA). Cells ($2x10^5$ cells/well) were seeded onto 12-well culture plates.

For the air/liquid interface culture, cells were grown on porous membranes (0.4 µM) in Snapwell inserts as shown in Figure.2.13. A transwell culture insert with a 12 mm diameter provided a growth area of 1.12 cm^2 supported by a detachable ring that was placed in a 12 well culture plate. Culture media supplemented with 5% FCS, 1% antibiotics and 0.01 M HEPES buffer was added to the membranes and the Snapwell inserts were incubated at 37ºC for 1 h as an initial equilibrium time to improve cell attachment. Culture media was then removed from the top and replaced with fresh medium (0.5 mL) containing the cell suspension (2×10^5 cells). Cell cultures were incubated at 37ºC in a humidified CO_2 incubator for 24 h. Before exposure, cell confluence (75-80%) and attachment were observed using the light microscope. The medium was removed from newly confluent cells and membranes washed with Hank's balanced salt solution (HBSS).

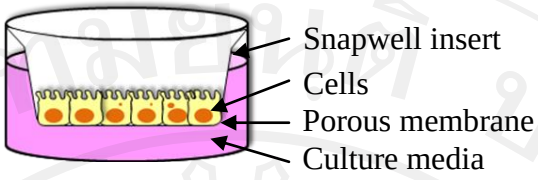


Figure 2.13 Cells were cultured by an air/liquid interface technique.

2.2.3 Toxicity Testing by using the Static Exposure to Alveolar Epithelial Cells

Cells cultured by both methods were exposed to benzene vapor by static exposure for 2 h. For conventional culture, cells were grown in a humidified 5% CO_2 incubator at 37 °C for 24 h after exposure. For the air/liquid interface culture, the inserts were transferred into conventional 12-well plates, and culture medium was added to the upper side of the Snapwell. Cells were incubated for 24 h in a humidified 5% CO_2 incubator at 37 °C. Cell viability was determined by Colorimetric XTT assay. Culture supernatant was collected for the quantification of IL-6.

2.2.4 Toxicity Testing by using the Developed Direct Alveolar Epithelial Cells - Air Exposure Prototype

Before testing, the Air Exposure chamber was sterilized by UV light and then the ALI cells were carefully placed in the exposure chamber. After that we turned on the pump and Air was into contact with the cells at different time (4, 6, 8, 12 hour 24 hours) for tested appropriate time. After exposure Cells cultured by air/liquid interface culture were exposed to Air by dynamic exposure different time (4, 6, 8, 12 hour 24 hours). For the air/liquid interface culture, the inserts were transferred into conventional 12-well plates, and culture medium was added to the upper side of the

Snapwell. Cells were incubated for 24 h in a humidified 5% CO_2 incubator at 37 °C. Cell viability was determined by Colorimetric XTT assay. Culture supernatant was collected for the quantification of IL-6 and IL-8, Next, we used the Air Exposure chamber to test on actual pollution sources. We selected three sources which are divided into two groups.

1. Test sites, were Warorot market Ampher Muang, Chiang Mai had high air pollution from traffic and grilled food and Saraphi District had high air pollution from traffic and the incidence of lung cancer is highest in Chiang Mai.
2. Control site was Phalad temple Ampher Muang, Chiang Mai. There are a lot of trees and low level of air pollution.

This study uses the Air Exposure chamber to tested at test site and control site in the same time.

2.2.5 Toxicity Testing by using the conventional method

2.1.4.1 Collection of PM

Particulate matter samples were collected using mini-volume portable air samplers (AIRmetrics MiniVol®portable air samplers, Springfield, OR 97477, USA, www.airmetrics.com) with cut points of 2.5µm, as described in previous research[83]. Samplers were operated at a flow rate of 5 L/min, and the flow rates were checked before and at the end of the study to ensure a constant flow rate throughout the sampling period. Samples were collected on 47 mm fiber-film filters (type T60A20, Pallflex Products Corporation, Putnam, CT, USA). A micrometric balance (Sartorius Ag, Germany), with accuracy of 0.001 mg, was used to weigh the filters. The filters

were conditioned in an electronic dessicator, with a temperature of 25°C and relative humidity of 50%, before and after sample collection. The balance was placed on an anti-vibration table, on top of a concrete bench

2.1.4.2 Preparation and extraction of filters

The fiber-coated filter was cut in small pieces and placed in a beaker with approximately 200 ml of dichloromethane. The filters were then sonicated in an ultrasonic bath for 15 minutes. The extract was filtered through Whatman No. 41 filter paper into a round bottom flask. Anhydrous sodium sulfate was put into the funnel before filtering in order to remove water from the sample. The sonication was repeated two more times with ~100 ml of dichloromethane. The beakers and funnels were rinsed with dichloromethane after subsequent filtration. The extract was evaporated by using a vacuum rotary evaporator to nearly dryness at 35°C, The residue was quantitatively resolved in culture media and passed through a Millipore filter membrane to get a sterile solution for use in the cytotoxicity test, IL-6 and IL-8 scretion. Extracts were stored in the dark at 4°C in sealed vials prior to the assays.

2.2.6 Colorimetric XTT assay

XTT (sodium-2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5- [(phenylamino) Carbonyl]-2H-Tetrazolium inner salt) cell viability assay was performed to assess the mitochondrial metabolic activity of the viable cells. Viable cells can convert a soluble tetrazolium salt to a formazan product. XTT dye and electron-coupling reagent were mixed in a ratio of 200:1. The XTT/electron-coupling reagent (500 µL) was added to the cells on top of the membrane and incubated at 37 °C in humidified 5% CO_2 for 4

hr. The solution was transferred into 96-well plates. The absorbance was measured using an ELISA plate reader at 450 nm.

2.2.7 Interleukin release assay

The cytokine IL-6 and IL-8 was chosen as a pro-inflammatory biomarker to evaluate the inflammatory response. Medium was collected and frozen at -70 °C before the analysis with an ELISA assay (Biolegend, USA). Capture Antibody was diluted in Coating Buffer and 100 µL of diluted Capture antibody solution were coated onto each well of 96-wells plate. The plate was covered and incubated overnight at 4°C. A standards and samples were run in triplicate. Standard curve is required for each assay. The plate was wash 4 times with at least 300 µL Wash Buffer per well and tapped upside down on an absorbent paper to remove residual buffer. To block non-specific binding and reduce background, add 200 µL of Blocking reagent per well. Plate was sealed and incubated at RT for 1 hour with shaking at 200 rpm on a plate shaker. Standard human IL-6 and IL-8 concentrations are range from 7.8-500 pg/mL and 7.8-1,000 pg/mL, respectively. One hundred microliters of standard dilutions and samples were added to the appropriate wells. Plate was sealed and incubated at RT for 2 hours with shaking. Plate was washed 4 times with Wash Buffer. One hundred microliters of diluted Detection Antibody solution was added to each well, plate was sealed and incubated at RT for 1 hour with shaking. Plate was washed 4 times with Wash Buffer. One hundred microliters of diluted Avidin-HRP solution was added to each well, seal plate and incubate at RT for 30 minutes with shaking. Plate was washed 5 times with Wash Buffer. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute. One hundred microliters of TMB

Substrate solution was added and incubated in the dark for 15-30 minutes or until the desired color develops. Positive wells should turn blue in color. It is not necessary to seal the plate during this step. Stop reaction by adding 100 μL of Stop Solution to each well. Positive wells should turn from blue to yellow. Read an absorbance at 450 nm within 30 minutes.

2.2.8 Statistical analysis

Statistical analyses were performed using SPSS program. Results were expressed as mean $\pm$ standard deviation for three different experiments. Mann-Whitney U test were used to determine the differences. P value less than 0.05 was considered as statistically significant.