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Cytotoxicity Studies on Combustion Gas of Polyvinyl Chloride (PVC) Resin

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ABSTRACT

In this study we used an oxygen-based bomb calorimetry to combust polyvinyl chlorine (PVC) resin under different oxygen-demanded pressure values, and the generated combustion gas was absorbed by water. Three cultured cells, human fetal lung tissue cell (MRC-5), African green monkey kidney cell (Vero), and Chinese hamster ovary cell (CHO), were used to determine the cytotoxicity of these water adsorbents. The number of dead MRC-5 cells was determined by MTT analysis and that of Vero and CHO were determined by ELISA analysis. Results show that of all the water adsorbents the Vero cell line is most sensitive to the cytotoxicity test. In addition, molecular chlorine was found to be the major toxicant in such a sample.

Keywords: Vero; CHO; MRC-5; Cell line; Cytotoxicity.

INTRODUCTION

Chlorinated plastics (such as polyvinyl dichloride, PVDC, and polyvinyl chloride, PVC) have been applied to many uses in people's life, due to the fact that PVC products possess several excellent properties: a turntable T_g range, the ability to easily form a thin film, forming and a good solvent resistance property. It is well known that these chlorinated plastic wastes in municipal solid wastes (MSW) and medical wastes are not suitable treated by incineration. PVC products should be separated from MSW and prohibited from use in the packaging of food and medical products. Although replacement materials of PVC have been developed for years, it seems there is no chance of totally replacing PVC by only one currently available plastic. Therefore, PVC based products are readily found in municipal solid wastes (MSW), and they comprise about 14% in the dry-based weight of the MSW of Taiwan.

The combustion of MSW containing PVC and NaCl in an oxygen insufficient state will generate dioxin-like compounds and chlorinated poly-aromatic compounds and molecular chlorine gas (McNeill *et al.*, 1995; Wey *et al.*, 2001; Huang *et al.*, 2006). Recently, studies on the cytotoxicity of PVC resin, additive and PVC combustion gas (Eskes *et al.*, 1999; Xu *et al.*, 2004) have also been reported. Due to the fact that 68% of the chlorine in the char derived from MSW is water-soluble (Huang *et al.*, 2006), the investigate of the contribution of molecular chlorine to the cytotoxicity of water absorption of PVC combustion gas might be helpful in clarifying the source of the toxicity in the flue gas of MSW combustion. In this study we used an oxygen-based bomb calorimetry (Albert, 1998) to combust PVC plastic under various conditions and let the combustion gas pass through a water absorbing beaker. The test of cytotoxicity of these absorbents was carried out on human fetal lung tissue cells (MRC-5), African green monkey kidney cells (Vero) and Chinese hamster ovary cells (CHO). The authors selected three cultured cells, human fetal lung tissue cell (MRC-5), African green monkey kidney cell (Vero) and Chinese hamster ovary cell (CHO), due to the transportation pathway of water soluble air pollutants in the flue gas of PVC combustion is through lung and dissolve in blood, finally go to kidney. Two examination methods are used, due to the sensitivity of cell death and survive of these cell lines.

EXPERIMENTAL SECTION

Jet Isothermal Bomb

Bomb calorimetry measurement is a fundamental experiment to determine combustion enthalpy (heat of combustion, dry-based higher heating value) of matter. Recently, a new calorimeter filled with oxygen was built based on a traditional instrument (Albert, 1998). Calorimetric measurements of polymers were conducted on a Gallenkamp No. CAB101.AB1.C oxygen bomb calorimeter made by Sanyo Co. The measured combustion heat was calibrated by the water equivalent of benzoic acid and was equivalent to the dry-based higher heating values $(HHV)_D$. PVC resin was charged from Chi-Ga Co. (in Taiwan) and was shredded by a crusher with steel knifes at the shedding rate of 3000 rpm. Chilly Mechanical Company in Taiwan made the crusher for this study. National Science Council of the Republic of China provided financial support for this research under contract number NSC 95-2221-E-020-023.

Brief operation procedures of the calorimeter were followed by the test procedures recommended by the manufacture as followings. The bucket of Sanyo Calorimeter, an isothermal jacket surrounding a combustion bomb, was filled with two liters of water; a water circulator circulated the water in order to keep a constant temperature of 24°C. The volume of the combustion bomb was 200 mililiters. One gram of polymer powder was massed and mixed carefully before being compressed by a compressor at a pressure of 10 MPa into the disc form with a 2 cm diameter pellet. Sample pellets were placed in the holder

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inside the bomb, which was sealed manually after which it was filled with oxygen at a pressure of 20-30 bar. 10-cm cotton wire was placed on the top of the pellet. The cotton was connected to a 10-cm nickel wire linked to the heating promoter. An electrical arc initiated the combustion of polymer samples, and the final exhaust gas was absorbed by 500 miniliter water under a flow rate of about 20 L/min at room temperature. The combustion heat of the polymer (HHV) was calculated from the temperature difference of the water in the bucket. Each sample was tested three times to obtain an average combustion heat with a low standard of derivation. The residual gas in the bomb was released into a beaker filled with water.

Cytotoxicity Test - MTT Method

Principle of MTT Testing Method (Chen *et al.*, 2006; Huang *et al.*, 2007)

In this paper, the cytotoxicity of all absorbents was carried out on MRC-5 cell line (Hsiao *et al.*, 2000) from lung tissue of fourteen-week old human fetal tissue and examined by MTS analysis. The cytotoxicity obtained from the absorbents was carried out on MRC-5 cell line and examined by MTS analysis. The principle of MTS analysis is to monitor the reduction percentage of R-tetrazolium into R-formazan and to quantify this by the spectrophotometer method at 492 nm. The cytotoxicity data is represented as the relative equivalent percentage (REP%) which is a relative ratio of the number of cell dead in 12.5 uM cumene-hydroperoxide solution to that of sample solutions. The IUPAC name of MTS is 3-(4, 5-dimethyl thiazol-2-yl)-5-3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium.

Cell Line Conditioning

Seven miniliter of each serious of absorbents (in water, acetone and cyclohexane) was firstly dried by a nitrogen-purge. The sample was then dissolved in 1 miniliter of dimethyl sulfoxide (DMSO) followed by filtration with a sterile 0.2 μm filter. The MRC-5 cell line (ATCC CCL-171, CCRC 60023) was purchased from the Institute of Food and Industrial Development in Taiwan, and was cultured in a 10% minimum essential medium (MEM) with 10% Fetal Bovine Serum. Cultures were transferred to a 96-well plate with a concentration of 10,000 cells per well and incubated at 37°C for 24 hours under an atmosphere of 5% CO_2. Then DMSO replaced the medium of the cells with various dilution ratios before sitting for another 24 hours. In all six wells of cultures were tested for each sample.

MTS Analysis

At the end of pretreatment, 20 μL of the MTS and PMS (phenazine methosulfate) mixture was added at a ratio of 1:20 to each well and incubated for 4 hours at 37°C, under an atmosphere of 5% CO_2. The appropriate amount of sodium dodecyl sulfate (SDS) was added into the final well, and the intensity of absorbency was recorded for each cell at 492 nm. A DMSO containing 2.5 μM of cumene-hydroperoxide solution was used as the reference. The cytotoxicity was calculated into the relative efficiency potential (REP%) :

REP% = [Intensity of sample treated cell – Intensity of pure DMSO treated well] / [Intensity of sterile DMSO treated cell – Intensity of pure DMSO treated well] (1)

Positive control data of MTS method is listed as following:

Cumene Hydroperoxide (μM)	REP%
2.5	23.2
6.0	54.2
12.5	100

Cytotoxicity Test - ELISA Method

Principle of Testing Method (Shih *et al.*, 2004; Huang *et al.*, 2008)

In this paper, the cytotoxicity of all extracts were performed on African green monkey kidney cells (called Vero cell) and examined by the Dynex-MRX Enzyme linked immuno absorbent assay (ELISA) reader (made by Dynex Technologies, USA). The principle of ELISA analysis is to analysis the lactate dehydrogenase (LDH) concentration released from Vero cells when they die. Fine chemicals of FBS, DMEM and TVS were obtained from HyClone Co. (USA) and the cytotoxicity detection kit (LDH) was from Roche Applied Science. The dilution of all extracted samples was by D.I. water.

Cell Line Conditioning

Vero cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 1% Fetal Bovine Serum (FBS) for a few days. Cultured cells were transferred to a 96-well plate with a concentration of 10,000 cells per well and continually incubated at 37°C for another 24 hr under an atmosphere of 5% CO_2. A 0.25% trypsin versens solution (TVS) was used as the digesting solution to separate the single cell layer from the bottom of the incubation bottle to form a suspended solution.

CHO cells were cultured in HAM medium (Nutrient Mixture F-12 HAM) with 10% Fetal Bovine Serum (FBS) for a few days. Cultured cells were transferred to a 96-well plate at a concentration of 10,000 cells per well and continually incubated at 37°C for another 24 hr under an atmosphere of 5% CO_2. A 0.25% trypsin versens solution (TVS) was used as the digesting solution to separate the single cell layer from the bottom of the incubation bottle to form a suspended solution.

Assay Procedures

The cell suspension solution was diluted by this medium to adjust the cell number to 1×10^5 cells 1/mL before it was seeded into a 96-well tissue culture microplate with 100 μL per well cell-suspension. Then the microplate was once again incubated overnight (37°C, 5% CO_2, 90% humidity). The adherent cells were exposed to samples for 2, 4 and 6 hr. Then the microplate was centrifuged for 5 minutes under a centrifuge file of 250 G. 100 μL of supernatant in each well was removed carefully and then transferred into the wells of an optical clear 96-well flat bottom microplate, and 100μL reaction mixture was added into each well. The reaction mixture was composed of 2-p-Iodophenyl-3-pnitrophenyl -5-phenyltetrazolium chloride (INT) dye solution and Diaphorase/NAD^+ mixture (as catalyst) in a ratio of 45:1 (V./V). The optical clear microplate was incubated for another 30 minutes at room temperature in a dark room before it was examined by an ELISA reader. The absorbency of the samples was measured by an ELISA machine at 490 nm to obtain an OD value from the absorbency of spectrophotometer. The negative control data of ELISA method is listed as following:

	OD
empty micro-plate (Blank)	0.037
1%DMEM with cell free	0.273
1%DMEM with cell seed (Low control standard)	0.389

The cytotoxicity (REP%) of all extracts was obtained through the following equation based on OD value of sample, high control standard and low control standard A 2% Triton-100 solution and 100 μL conditioning medium (DMEM for Vero cell and HAM for CHO cell) solutions were used as the high and the low control standard, respectively.

$$\text{REP\%} = 100 \times [\text{Sample} - \text{Low contral}]/[\text{High control} - \text{Low control}] \quad (2)$$

And the positive control data ELISA method is listed as following:

Triton solution (%)	OD
0.5	2.626
1	2.708
2 (High control standard)	2.851

Determination of Concentration of Molecular Chlorine (Cl_2) in Absorbents by Amperometric Titration

Because the dechlorination of chlorinated organic compounds, vaporization of metallic chlorates, and degradation of HCl from fly ash usually produce Cl_2, ClO_2, Cl_2O, Cl_5O_7 and Cl in the emitted gases, under high temperatures chlorine (Cl_2) has a higher solubility in chloroform or CCl_4 than that in water, and lower solubility of 0.9972 g in 100.0 g water at 10°C, i.e., 9997.2 ppm. An automatic amperometric titration system (Autop CAT^{TM} 900, Hach Company, USA) was used to determine the concentration of molecular chlorine (Cl_2) in each absorbent sample.

Following are the operation procedures for this machine: 1 mililiter of diluted absorbent material was placed in a beaker. Then it was mixed with 1.0 mililiter of phosphate-based buffer solution (pH 7.0) for 4 seconds. Finally the mixture was titrated by a phenylarsenic oxide (PAO, a reductant) solution to determine the concentration of free chlorine from the electrical potential curve.

Determination of Total Carbon (TC), Inorganic Carbon (IC), and Total Organic Carbon (TOC) in Absorbents

The TOC test was performed by the Shimadzu TOC-5000 instrument. Potassium hydrogen phthalate, sodium carbonate, and sodium hydrogen carbonate were used as standards for TOC and IC measurements. The maximum heating temperature was set to 680°C for a TOC test.

RESULTS

The combustion of PVC may generate HCl, Cl_2, and chlorinated organic compounds. Table 1 lists TC, IC, and TOC values of water adsorbents of PVC combustion. The amount of oxygen demanded was calculated from the bomb pressure to be 0-20 mg of O_2 per gram f PVC resin. The cytotoxicities of the obtained water adsorbents in different dilution ratios from the MRC-5/MTT test, Vero/ELISA test, and CHO/ELISA test I have been translated into AREP% and listed in Table 2. It is clear to observe that these three cells all indicate different cytotoxicity for the same sample. But only the Vero cell shows a linear dependence to the chemical composition of the water-absorbing sample. Fig. 1 describes a good regression between experimental data and calculated data by following Eq. (3) (expected for the data of zero oxygen demanded). In this study, we also formulated an equation for Vero cells:

$$\text{AREP\%} = 4.5 \times [0.75 \times (\text{TotalCl}_2/\text{TotalTOC}) + 0.25 \times (\text{TotalCl}_2/\text{TotalIC}) - 1] \quad (3)$$

DISCUSSION

The obtained TC values in Table 1 were about 4-27 mg/L. It is 3.3-26 mg/L for the IC value and 0.67-2.6 mg/L for TOC value, pH Values of Cl_2 were 2-7.0 and 8-43 mg/L, respectively. Data shows that the highest generation rate of Cl_2 was observed at 12 mg g × 1/PVC. The meaning of the combustion test characterized by zero mg oxygen per gram PVC sample is that the PVC was combusted under 1 atm of air in the bomb.

Table 1. TC, IC and TOC values of Water Adsorbent of PVC Combustion.

Oxygen Demanded	TC (mg/L)	IC (mg/L)	TOC (mg/L)	pH	Cl_2 (mg/L)
0 mg-O_2/g-PVC	4.079	3.377	0.67	7	0
4 mg-O_2/g-PVC	19.56	18.80	0.76	2.06	8
8 mg-O_2/g-PVC	27.53	26.08	1.45	2.68	40.9
12 mg-O_2/g-PVC	26.79	24.68	2.11	2.93	43.5
16 mg-O_2/g-PVC	23.20	20.59	2.61	3.94	25.4
20 mg-O_2/g-PVC	23.60	22.66	0.94	4.16	28

Table 2. Averaged Cytotoxicity data of Water Adsorbent of PVC combustion Gas by Various Cell Lines.

Oxygen Demanded	MRC-5	Vero	CHO
0 mg-O_2 g × 1/PVC	-6.55	113.83	5.65
4 mg-O_2 g × 1/PVC	-54.5	54.87	-5.72
8 mg-O_2 g × 1/PVC	4.00	122.82	5.83
12 mg-O_2 g × 1/PVC	7.55	122.23	-8.83
16 mg-O_2 g × 1/PVC	6.5	39.56	20.55
20 mg-O_2 g × 1/PVC	7.6	83.53	90.2

(unit: %)

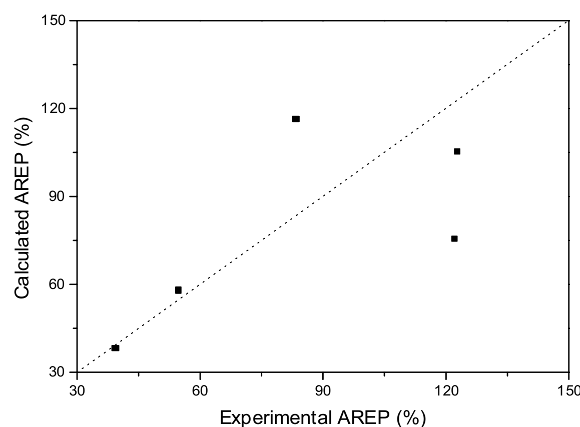

Fig. 1. Comparison of experimental and calculated AREP% for Vero Cell.

In this paper we use a new toxicity indicator, the averaged relative efficiency percentage (AREP%) (Huang and Shue, 2007) to compare the values of cytotoxicity from different experimental conditions. AREP% is obtained from the intercept value of the plot of REP% versus dilution ratios. The AREP is meaning the real cytotoxicity of undiluted water adsorbent. This equation is as similar as our previous work on the cytotoxicity of the exhaust gas from a heating municipal solid waste baghouse ash (Huang and Shue, 2007).

And this equation also reflects some information: (1) the final AREP value of water adsorbent could be reduced by TOC, and (2) molecular chlorine would cause a high cytotoxicity.

CONCLUSIONS

Our experiment shows that the Vero cell line is more sensitive

to the cytotoxicity test of the water adsorbents than MRC-5 cell, and molecular chlorine is the major toxicant in such a sample.

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