Characterisation of a Vitrocell® VC 10 exposure system using dose tools and biological responses *in vitro*

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Introduction

•Traditional smoke exposure techniques are based on the particulate phase of tobacco smoke and not the complete whole smoke aerosol. The particulate phase of tobacco smoke makes up approximately 5-10% whereas the vapour phase makes up the remaining 90-95% (v:v)[1].

•To overcome these challenges, whole smoke exposure systems have been developed that expose cell cultures to diluted tobacco smoke and capture the full interactions of both smoke phases.

Aim

To characterise a Vitrocell® VC 10 smoke exposure system using a battery of tests. We achieved this by measuring smoke deposition[2] in relationship to airflow (L/min); vapour phase dilution using a known smoke marker carbon monoxide (CO). Finally we assessed biological responses using the Ames and Neutral Red uptake (NRU) assays.

Materials and Methods

Cigarette Smoke Generation

Smoke was generated using a Vitrocell® VC 10 Smoking Robot, (Vitrocell® Systems, Waldkirch, Germany). Dilutions were achieved by diluting smoke in air (L/min), with a vacuum of 5ml/min/well. 3R4F cigarettes were smoked to the ISO smoking regime (35ml puff over 2 seconds, once a min - ISO 3308:1991).

Measurement of Particulate Dose

Four quartz crystal microbalances (QCM) were installed into a 6PT-CF Vitrocell® exposure module to assess particulate dose[3]. During biological exposure, a QCM, located in position 4, recorded real-time *in situ* deposition data, which allowed data to be presented as a function of deposited mass **(Fig. 1-2)**.

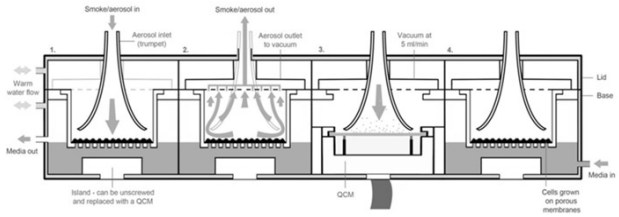

Figure 1: Schematic cross-section of the 6PT-CF mammalian exposure module depicting how smoke is delivered to cell culture inserts at the air-liquid interface (ALI) and the QCM module[3]. A similar setup, without media, was used for Ames agar plates.

Measurement of Carbon Monoxide Concentration

CO concentrations were determined via the analysis of the diluted cigarette smoke, collected from exhaust in Douglas gas bags, using a Signal® 7000-FM gas analyser **(Fig. 3)**.

NRU Assay

BalB/c 3T3 cells were seeded into 24 mm Transwells® and maintained in culture for ~24 h prior to exposure to achieve near-confluent monolayers. Cells were exposed at the ALI to freshly generated cigarette smoke. Following exposure (184 min), cells were assessed for cytotoxicity in the NRU assay **(Fig. 4)**.

Ames Assay

Salmonella typhimurium (YG1042) were plated onto 35 mm Vogel-Bonner agar plates in the presence of 10% S9 using a spread plate methodology. Bacterial agar plates were smoke exposed for 24 min, incubated at 37°C for 3 days and revertant colony numbers determined by automated scoring **(Fig. 5)**.

Results

Measurement of Particulate Dose

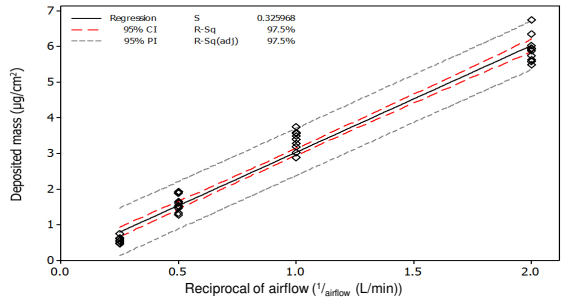

Figure 2: Measurement of deposited particulate mass from a 24 minute smoke exposure at four smoke dilutions (Airflows 0.5, 1.0, 2.0 and 4.0 L/min). Results are presented as a reciprocal of the airflow ($^1/_{airflow}$ (L/min)) and are based on three independent experiments. A regression fit correlation of R^2 = 0.975 was observed with 95% confidence intervals (red dash) and probability intervals (grey dash).

Measurement of CO Concentration

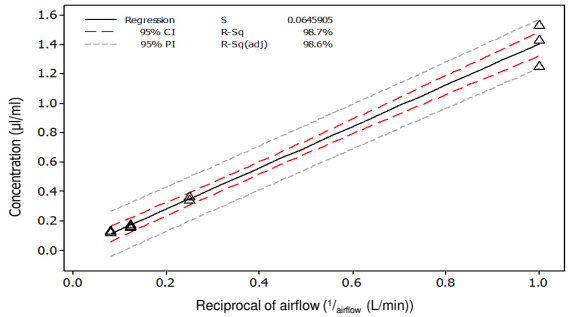

Figure 3: Measurement of CO concentrations following a 16 minute smoke exposure at four smoke dilutions (Airflows 1.0, 4.0, 8.0 and 12.0 L/min). Results are presented as a reciprocal of the airflow ($^1/_{airflow}$ (L/min)) and are based on three independent experiments. A regression fit correlation of R^2 = 0.975 was observed with 95% confidence intervals (red dash) and probability intervals (grey dash).

NRU Assay Data as a Function of Deposited Mass

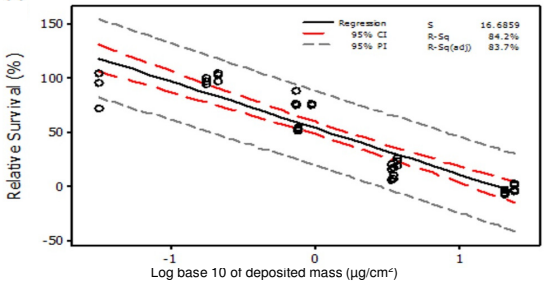

Figure 4: NRU assay relative survival data (v. air control) presented as a function of deposited mass (obtained *in situ* of exposure) on a log scale with confidence intervals (red dash) and probability intervals (grey dash) of 95%. A correlation of R^2=0.84 was observed between deposited mass and cytotoxicity. Results are based on three independent experiments. Airflows = 1.0, 4.0, 8.0 and 12.0 L/min.

Ames Assay Data as a Function of Deposited Mass

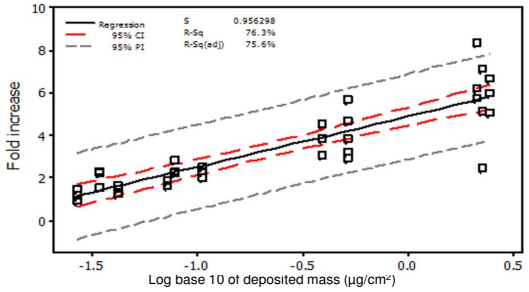

Figure 5: Ames assay fold increase data presented as a function of deposited mass (obtained *in situ* of exposure) on a log scale with confidence intervals (red dash) and probability intervals (grey dash) of 95%. A correlation of R^2=0.76 was observed between deposited mass and mean fold increase in revertant numbers. Results are based on three independent experiments. Airflows = 1.0, 4.0, 8.0 and 12.0 L/min.

		NRU Correlation of R^2 = 0.847		AMES Correlation of R^2 = 0.763		
Airflow (L/min)	Reciprocal of airflow (^{1}/airflow (L/min))	Mean deposited mass for NRU exposure (µg/cm² ± STDV)	Mean % relative cell survival ± STDV	Mean deposited mass for Ames exposure (µg/cm² ± STDV)	Mean revertant fold increase ± STDV	Mean total revertants ± STDV
1.0	1.000	22.8 ± 1.7	-2.5 ± 3.3	2.30 ± 0.14	5.9 ± 1.6	78.6 ± 20.6
4.0	0.250	3.5 ± 0.1	16.7 ± 7.4	0.50 ± 0.10	4.0 ± 0.9	53.1 ± 9.6
8.0	0.125	0.8 ± 0.1	69.9 ± 13.0	0.09 ± 0.02	2.2 ± 0.4	30.2 ± 4.1
12.0	0.080	0.1 ± 0.1	96.8 ± 10.1	0.03 ± 0.01	1.6 ± 0.5	21.2 ± 5.0

Table 1: Raw data values including airflows, reciprocal of airflows, correlations, standard deviation between experiments, relative survival, mean revertant and fold increase numbers and *in situ* obtained deposited mass.

Conclusions

•Correlations between airflow (L/min) and deposited mass and CO were observed, adding confidence to the exposure system:-

- **Deposited mass correlation with airflow, R^2 = 0.97**
- **CO concentration correlation with airflow, R^2 = 0.98**

•The study demonstrated consistent responses using 2 independent biological assays for the assessment of cigarette smoke cytotoxicity (NRU) and mutagenicity (Ames)

•Biological results have been presented as a function of deposited mass obtained *in situ* of exposure, giving our data a gravimetric measure. Correlations with deposited mass for both systems were observed:-

- **NRU correlation with deposited mass, R^2 = 0.84**
- **Ames correlation with deposited mass, R^2 = 0.76**

•As CO is only one smoke marker and may not be representative of the complete aerosol, additional thoughts should be given to alternative gas phase dose tools

•It is likely that both fractions of cigarette smoke play an important role in biological responses and therefore it is important to characterise these within an *in vitro* system

•This study has increased our knowledge and working understanding of the VC 10 exposure system, but further work is required to fully characterise this system

References

1. Clunes, L., Bridges, B., Alexis, N., Tarran, R. *In vivo* versus *in vitro* airway surface liquid nicotine levels following cigarette smoke exposure. Journal of Analytical Toxicology 2008; 32: 201–207.
2. Adamson, J., Hughes, S., Azzopardi, D., McAughey, J., Gaca, M. Real-time assessment of cigarette smoke particle deposition *in vitro*. Chemistry Central Journal 2012; 6:98.
3. Adamson, J., Thorne, D., Dalrymple, A., Dillon, D., Meredith, C. Cigarette smoke deposition in a Vitrocell® exposure module: real-time quantification *in vitro* using quartz crystal microbalances. Chemistry Central Journal 2013, 7:50

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