Assessment of dosimetry and biological responses *in vitro* using a Vitrocell® smoke exposure system

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Introduction

The development of whole smoke exposure systems has been driven by the fact that traditional smoke exposure techniques are based on the particulate phase of tobacco smoke and not the complete whole smoke aerosol. The particulate phase of a 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 which expose cell cultures to diluted tobacco smoke and capture the full interactions of both smoke phases.

Aim

In this study we characterised a whole smoke exposure system with relation to airflow dilutions (L/min); using deposited mass[2] and a known smoke marker carbon monoxide (CO). We also assessed biological responses using two biological systems, 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 minute - ISO 4387:1991).

Measurement of Particulate Dose

Four quartz crystal microbalances (QCM) were installed into a 6PT-CF Vitrocell® exposure module for assessment of 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)**.

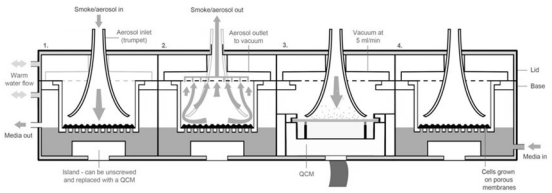

Figure 1: A schematic cross-section of the 6PT-CF mammalian exposure module depicting how smoke is delivered to cell culture inserts at the air-liquid interface (ALI) and the QCM module[3]. A similar setup, without media, was used for exposure of Ames agar plates.

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 24mm Transwells® and maintained in culture for approximately 24 hours prior to smoke exposure to achieve near-confluent monolayers. Cells were exposed at the ALI to freshly generated cigarette smoke. Following exposure (184 minutes) cells were assessed for cytotoxicity in the NRU assay **(Fig 4)**.

Ames Assay

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

Results

Measurement of Particulate Dose

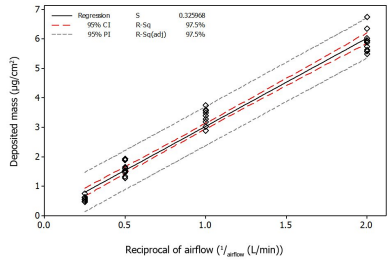

Figure 2: Measurement of deposited particulate mass from a 24 minute smoke exposure at four smoke dilutions (Airflows 0.5, 1.0, 2.0 and 4.0 L/min). Results are presented as a reciprocal of the airflow ($^1/_{airflow}$ (L/min) and are based on three independent experiments. A regression fit correlation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

Measurement of CO Concentration

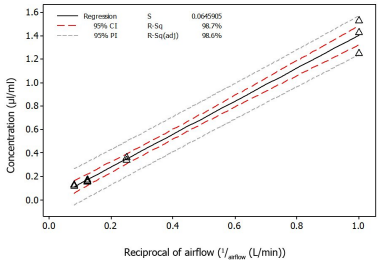

Figure 3: Measurement of CO concentrations following a 16 minute smoke exposure at four smoke dilutions (Airflows 1.0, 4.0, 8.0 and 12.0 L/min). Results are presented as a reciprocal of the airflow ($^1/_{airflow}$ (L/min) and are based on three independent experiments. A regression fit correlation of 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

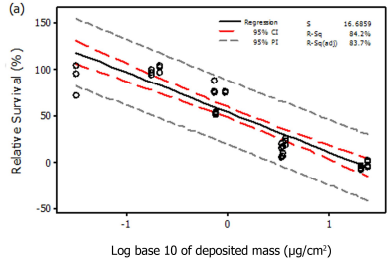

Figure 4: NRU assay relative survival data (v. air control) presented as a function of deposited mass (which was obtained *in situ* of exposure) on a log scale with confidence intervals (red dash) and probability intervals (grey dash) of 95%. A correlation of R^2=0.84 was observed between deposited mass 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

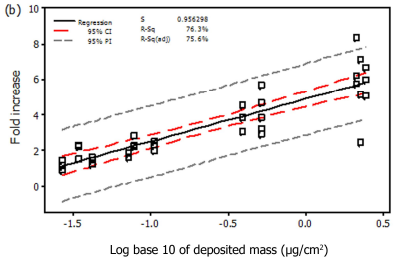

Figure 5: Ames assay fold increase data presented as a function of deposited mass (which was obtained *in situ* of exposure) on a log scale with confidence intervals (red dash) and probability intervals (grey dash) of 95%. A correlation of R^2=0.76 was observed between deposited mass and mean fold increase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

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.

		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

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 two 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 consideration should be given to alternative vapour phase dose tools.
- We believe 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

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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.
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ABSTRACT

Routine toxicological assessment of tobacco smoke commonly uses the particulate fraction of the smoke aerosol. The particulate phase of cigarette smoke makes up a small percentage of the total aerosol, approximately 5-9% by weight. The remaining ~91% is associated with the vapour phase of cigarette smoke and is not routinely evaluated. Whole smoke exposure systems are capable of capturing the full interactions of both the particulate and vapour phase together and offer unique potential for toxicological assessment.

In this study we used a Vitrocell VC 10 smoking robot to expose cell cultures at the air-liquid interface to ISO mainstream 3R4F cigarette smoke. All experiments were independent and completed a minimum of three times. For biological assessment we developed the Neutral Red Uptake (NRU) assay with a Balb/c cell line, and the Ames assay using bacterial strain YG 1042. We also used a novel quartz crystal microbalance (QCM) tool for real-time *in situ* deposition analysis. For the Ames assay we observed a mean fold increase of 5.9 at the highest concentration of smoke tested (1.0 L/min), which correlated with a 2.29 µg/cm^2 increase in particulate deposition. Clear differences were seen at all doses; for example, 12.0, 8.0 and 4.0 L/min produced a 1.6, 2.2, 4.0 and 0.03, 0.09, 0.50 fold increase and deposited mass (µg/cm^2) respectively. The NRU assay showed a complete cytotoxic dose response (12.0 -1.0 L/min), with a calculated dilution IC_{50} and deposited mass IC_{50} of approximately 6.5 L/min and 1.7µg/cm^2 respectively.

We conclude that the VC 10 can be used in conjunction with routine toxicological assays for the assessment of cigarette smoke toxicity, as demonstrated by consistent responses in two independent *in vitro* test systems. Furthermore, QCM measurements *in situ* of exposure have enabled us to present biological data as a function of deposited mass. QCMs have also acted as an important QC tool for smoke exposure and provide valuable information on the exposure system itself.

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