Biological Test procedure for fresh generated smoke and aerosols

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1. Introduction

Smoke / aerosol contain particulate matter / droplets in the solid phase as well as volatile substances in the gas phase. Both fractions are of potential toxicological relevance and should be considered when assessing biological activity of fresh smoke / aerosol mixtures. Rapid ageing of the mixtures lead to problems regarding trapping and effective delivery of both fractions to the cells. Therefore, dedicated exposure systems have been developed allowing the exposure of biological *in vitro* systems to freshly generated smoke / aerosols.
All the presented methods are ISO17025 accredited in the ITL-Lab in Hamburg/Germany.

2. Materials and Methods

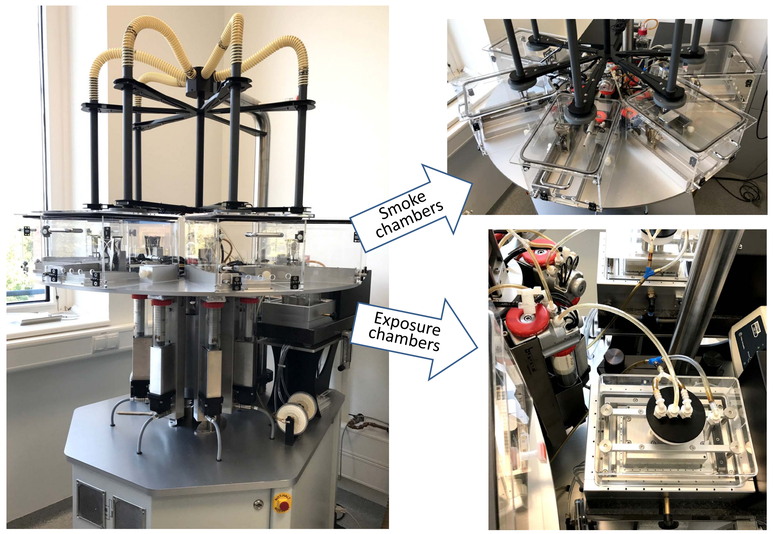
2.1 Tested products (TP)
Reference Cigarette 3R4F, Burley, Virginia and E-cigarettes with liquids containing 2.4% nicotine (w/w)

2.2 Smoke / aerosol generation
Fresh smoke was generated under ISO 3308 (35mL/2s/60s) bell-shape puff profile
Aerosol was generated under CRM N°81 (55mL/3s/30s) square wave puff profile

2.3 Cell exposure containers
96 MWP - Nunclon™ Delta Surface (#168136)
24 MWP - FALCON #353047 assembled with INSERTs MD24 0,4 (#NUNC 40620)

2.4 Exposed cells and bacteria
NRU assay - Human bronchia cells (BEAS-2B, ECACC 95102433)
IVM assay - Hamster Chinese lung cells (V79, ECACC 86041102)
Ames test – *Salmonella typhimurium* TA100 and TA98 (MOLTOX)

2.5 *In vitro* exposure systems
- Air Liquid Interface (ALI) exposure - Smoke Aerosol Exposure *In Vitro* System (SAEIVS) designed to expose cells in multiwell plates (MWP) and inserts / transwells [1].
- Bubbling Exposure – Smoke / aerosol bubbling system designed for direct bacteria exposure in 10 ml of PBS. 150 µl of the bacteria suspension is sampled between the smoke runs for analysis in Ames test.



3. ALI Exposure system

SAEIVS is designed to expose cells in MWP under ALI conditions. The system has been proven for its efficiency regarding biological effects especially induced by the gaseous components using appropriate positive controls. Up to 5 tobacco products or e-cigarette devices can be puffed simultaneously and deliver the smoke/aerosol in undiluted or diluted form to defined rows of wells. Separate row of wells were covered after defined number of puffs. Two exposure chambers with separate dilution systems allow parallel exposure to the same smoke / aerosol and their gas vapour phase in different dilution levels per each plate. Furthermore, the separate chambers enable testing of the same product in 2 different *in vitro* assays in different MWPs.

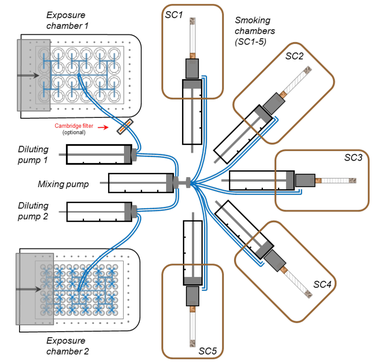
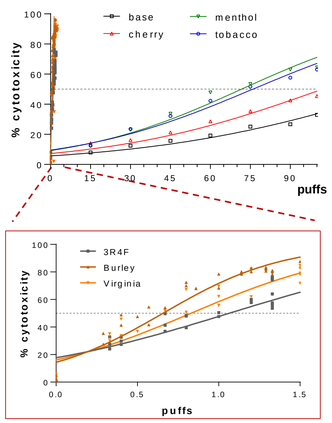


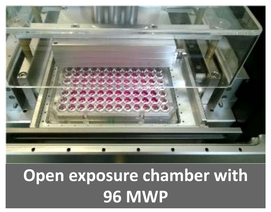
Fig1. Drawing of the SAEIVS

Neutral Red Uptake assay with BEAS-2B cells



The cytotoxicity of the products is tested by exposure of BEAS2B cells on 25 µl of collagen I matrix in 96 MWPs. The collagen layer guarantees stable exposure conditions over an extended time period sufficient for testing of several hundred of puffs. The BEAS2B cells are incubated over 3-4 cell divisions (65 hours) after the exposure covered by serum free medium. The low cell density allow constant growth of the cells over the whole incubation time.

The smoke was diluted by mixing of the smoke with air. The volume of smoke/aerosol distributed over the MTP correspond to the initial puff of 1 product. The excess volume was discarded.

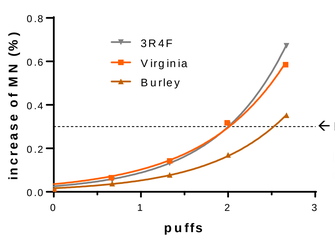


Open exposure chamber with 96 MWP

The EC50 of aerosol from e-cigarettes is about 100 fold higher than that of smoke.

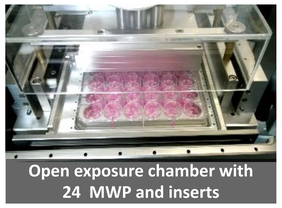
Micronucleus assay with V79 cells

The genotoxicity testing is performed with V79 hamster lung cells grown and exposed on inserts placed in 24 MWPs. After 20 hours incubation time the increased number of MN-cells was counted.



EC-MN (3-fold increase of MN) is the number of puffs which results in 3-fold increase against background level of micronuclei [2]

No effect were found after 100 puffs of aerosol exposure of e-cigarettes.



Open exposure chamber with 24 MWP and inserts

4. Bubbling Exposure system

Three-port adapter allow to bubble the smoke of up to 3 cigarettes successively through the impinger. After each puff a puff of air follows.
This test procedure is optimized for Ames testing with smoke/aerosol and their gas phase components in particular

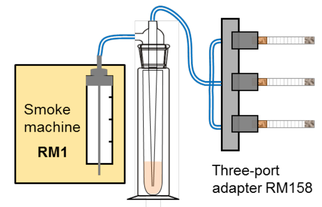
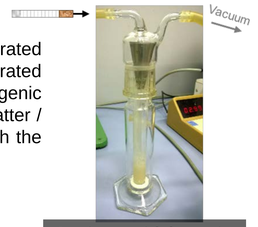


Fig2. Drawing of the bubbling exposure system

Ames test with *S. typhimurium*

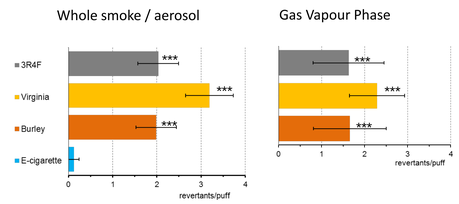
Mutagenicity testing is realized by bubbling 10-fold concentrated *S typhimurium* suspension in PBS directly with freshly generated smoke/aerosol. The strain TA100 shows sensitivity to mutagenic substances in both the gas phase and in the particulate matter / droplets. The strain TA98 shows only a weak response with the gas vapour phase.

Bactria for the Ames test were taken after defined number of puffs.

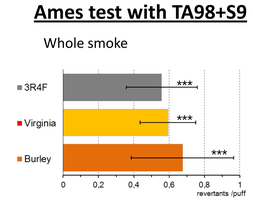


Impinger with bacteria suspension in tube

Ames test with TA100+S9



Ames test with TA98+S9



Less than 30 puffs of cigarettes deliver significant positive effects.
No effect were found with e-cigarette after 300 puffs.

5. Summary

- SAEIVS enables *in vitro* testing of aerosols generated from different product categories encompassing tobacco products and e-cigarette devices. Parallel testing in NRU and IVM assay as well as whole aerosols and their gas phase by using different dilution factors were performed.
- NRU assay - BEAS-2B cells seeded on a collagen-I matrix in a 96 MWP allow prolonged exposure to smoke and aerosol under ALI conditions
- IVM assay – V79 cells seeded on inserts or transwells in a 24 MWP allow exposure to smoke/aerosol under ALI conditions
- The sensitivity of fresh smoke/aerosol *in vitro* test systems towards gas phase components is of crucial importance

➢ For more details regarding the testing strategy of TPM, e-liquids and NTMs of Imperial Tobacco please refer to poster **P284**

References

1. Behrsing et al. (2017).*In vitro* exposure systems and dosimetry assessment tools for inhaled tobacco products: Workshop proceedings, conclusions and paths forward for *in vitro* model use. Altern Lab Anim. 45(3):117-158.
2. https://www.coresta.org/abstracts/optimisation-vitro-standard-testing-e-vapour-and-heated-tobacco-products-28177.html

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