Toxicology in Vitro xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

A new fluorescence-based method for characterizing *in vitro* aerosol exposure systems

Sandro Steiner, PhD *, Shoaib Majeed, MSc, Gilles Kratzer, MSc, Julia Hoeng, PhD, Stefan Frentzel, PhD

Philip Morris International R&D, Philip Morris Products S.A. (part of Philip Morris International group of companies), Quai Jeanrenaud 5, CH-2000, Neuchatel, Switzerland

ARTICLE INFO

Article history:
Received 25 July 2016
Received in revised form 9 September 2016
Accepted 20 September 2016
Available online xxxx

Keywords:
In vitro aerosol exposure
Aerosol exposure system characterization
Aerosol dosimetry
Vitrocell

ABSTRACT

Knowledge of how an *in vitro* aerosol exposure system delivers a test aerosols to the biological test system is among the most crucial prerequisites for the interpretation of exposure experiments and relies on detailed exposure system characterization. Although various methods for this purpose exist, many of them are time consuming, require extensive instrumentation, or offer only limited ability to assess the performance of the system under experimental settings. We present the development and evaluation of a new, highly robust and sensitive fluorometry-based method for assessing the particle size specific delivery of liquid aerosols.

Glycerol aerosols of different mean particle sizes and narrow size distributions, carrying the fluorophore disodium fluorescein, were generated in a condensation monodisperse aerosol generator. Their detailed characterization confirmed their stability and the robustness and reproducibility of their generation. Test exposures under relevant experimental settings in the Vitrocell® 24/48 aerosol exposure system further confirmed their feasibility for simulating exposures and the high sensitivity of the method.

Potential applications of the presented method range from the experimental confirmation of computationally simulated particle dynamics, over the characterization of *in vitro* aerosol exposure systems, to the detailed description of aerosol delivery in test systems of high complexity.

1. Introduction

With the wide commercialization of electronic cigarettes (e-cigarettes), there is a growing interest in evaluating the toxicity of the aerosols they generate (Farsalinos & Polosa, 2014; Riker et al., 2012; Pepper & Eissenberg, 2014; Pisinger & Døssing, 2014), which, in light of attempts to reduce animal experimentation, is projected to involve more and more *in vitro* toxicological studies. However, *in vitro* toxicological assessment of aerosols is technically challenging. It is generally accepted that exposing submersed cell cultures to extracts of aerosols trapped on filters or in solvents cannot, or can only to a very limited extent, simulate the interaction between a native aerosol and the epithelial lining of the respiratory tract, as the collection and processing of a test aerosol inevitably results in changes to its physicochemical properties. In addition, it is well established that the submersed state of cell cultures is not representative of conditions in the respiratory tract (Paur et al., 2008; Panas et al., 2014; Hirsch et al., 2013; Paur et al., 2011; BéruBé et al., 2010).

In consequence, aerosol exposure at the air-liquid interface (ALI) is the preferable approach. This method requires the test aerosols to be conditioned to meet specific requirements of the cell cultures in terms of temperature and humidity, and to be diluted to achieve realistic doses and dose responses (Paur et al., 2008; Grass et al., 2010). This is commonly achieved in aerosol exposure systems, several of which have been developed in the past years (Paur et al., 2008; Thorne & Adamson, 2013). However, whereas their working principles may vary greatly, they share the common property that besides adjusting the relative humidity, temperature and concentration, they potentially also change other aerosol characteristics. This includes, for instance, the particle number-size distribution (Alonso et al., 1999), the total particle mass (Chang et al., 1985) and the partitioning of semi-volatile compounds between particulate matter (PM) and the gas-vapor phase (GVP) (Chang et al., 1985). As the delivery efficiencies of GVP, PM and particles of different sizes must be assumed to not be equal (Fujitani et al., 2015; Ishikawa et al., 2016; Guha, 2008; Sahu et al., 2013), this inevitably influences the aerosol delivery to the biological test system. Predictions of dosing from the physicochemical properties of the original aerosol are therefore highly error-prone, and affect the relevance of *in vitro* studies that require the achieved aerosol delivery to be known, and moreover, to be representative for the targeted region of the respiratory tract (Ishikawa et al., 2016; Heyder, 2004). Furthermore, a fully reproducible performance of the exposure systems cannot be assumed by implication and in the case of systems that allow exposing multiple cell cultures simultaneously—be it as replicates or for testing

Abbreviations: CMAG, condensation monodisperse aerosol generator; DSF, disodium fluorescein; VC24/48, Vitrocell® 24/48 aerosol exposure system; PBS, phosphate buffered saline; PM, particulate matter; GVP, gas-vapor phase; APS, aerodynamic particle sizer; e-cigarettes, electronic cigarettes; GSD, geometric standard deviation; CFP, cambridge filter pad; CV, coefficient of variation; ULOQ, upper limit of quantification; LLOQ, lower limit of quantification.

* Corresponding author.
E-mail address: Sandro.Steiner@pmi.com (S. Steiner).

http://dx.doi.org/10.1016/j.tiv.2016.09.018

serially diluted aerosols (or both)—inaccurate dosing and/or non-uniform aerosol delivery may occur (Adamson et al., 2014).

Literature is available on how various aerosols, including smoke generated by conventional cigarettes, are delivered in *in vitro* exposure systems (Fujitani et al., 2015; Ishikawa et al., 2016; Adamson et al., 2014; Majeed et al., 2014; Kaur et al., 2010; Adamson et al., 2012; Asimakopoulou et al., 2011; Aufderheide et al., 2013; Kim et al., 2013; Scian et al., 2009; Tippe et al., 2002; Wiegand et al., 2015; Fröhlich et al., 2013; Mertes et al., 2013), but only a limited number of these studies focused on water-soluble, liquid aerosols containing low amounts of volatile and semi-volatile compounds, such as those generated by various e-cigarettes (Hajek et al., 2014). In many aspects, such aerosols behave differently than dry powders or combustion products (Koehler et al., 2012; Feng et al., 2015; Marple et al., 1991; Lízal et al., 2010; Mullins et al., 2003), and will as a result be delivered differently in *in vitro* exposure systems. In particular, if a direct toxicological comparison between whole smoke generated by conventional cigarettes and aerosols generated by e-cigarettes is attempted, great care must be taken when establishing comparable doses. Identical or nearly identical protocols for the generation and application of conventional cigarette smoke and e-cigarette aerosol as applied in many studies (Cervellati et al., 2014; Neilson et al., 2015; Scheffler et al., 2015; Shen et al., 2016) cannot be assumed to result in comparable delivery, unless this was confirmed by quantification of aerosol delivery to the exposure chambers or by detailed exposure system characterization.

Exposure system characterization can be approached in two distinct but not exclusive ways: By comparing the aerosol entering the system (or a part of the system) with the aerosol leaving the system (or a part thereof), or by direct quantification of aerosol deposition on relevant internal surfaces of the system. The former approach allows measuring aerosol evolution and deposition on-line, even simultaneously with the exposure of the biological test systems. It suffers, however, from low spatial resolution, as the exact location within the system at which the observed changes arise cannot be determined. Direct quantification of aerosol deposition, although it can be performed on-line (Thorne & Adamson, 2013; Adamson et al., 2012; Wiegand et al., 2015), is usually performed after exposure or in separate experiments specifically dedicated to system characterization. Commonly applied methodologies include for example counting deposited particles using electron microscopy (Panas et al., 2014; Fujitani et al., 2015; Kim et al., 2013; Tippe et al., 2002; Fröhlich et al., 2013), absorbance/colorimetric measurements (Majeed et al., 2014; Scian et al., 2009), or chromatographic procedures (Thorne & Adamson, 2013; Ishikawa et al., 2016; Majeed et al., 2014; Mertes et al., 2013). These methods allow the description of aerosol delivery in an exposure system with an impressive level of detail, and are clearly indispensable for describing the exact dosing of complex multicomponent aerosols. The advantages notwithstanding, these methods are usually time-consuming, expensive, and require extensive analytical equipment and may therefore not be suitable for many institutions performing *in vitro* aerosol exposures. In addition, in many aspects it is advantageous to perform exposure system characterization with model aerosols of low complexity, especially when a mechanistic understanding of the system and not only the aerosol delivery to the exposure chambers is of interest.

In this paper, we describe a method for the quantification of aerosol deposition that, because of its simple but robust character, allows characterizing the aerosol delivery in an aerosol exposure system in detail even if extensive analytical equipment is unavailable. We focused specifically on aerosols comparable to those generated by common types of e-cigarettes, because for this kind of aerosols, only a limited number of established methods for exposure system characterization are available. Since the aerosols generated by e-cigarettes (but also by various medical devices for drug inhalation) contain large amounts of glycerol (Hajek et al., 2014; Watts et al., 2008; Patton & Byron, 2007; Haghi et al., 2014), we chose glycerol as the aerosol material. Fluorometry was used for aerosol detection because of its robustness and simplicity in implementation, disodium fluorescein (DSF) was chosen as the fluorescent label.

As a proof of concept, we applied the method to the Vitrocell® 24/48 aerosol exposure system (VC24/48, VITROCELL Systems GmbH, Waldkirch, Germany), the system we use at our facility for exposing organotypic cell cultures as a part of the toxicological assessment of conventional and novel tobacco products. We thereby focused on the sensitivity of the method and on the dosing accuracy.

2. Materials and methods

2.1. Aerosol generation

Fluorescent aerosols were generated in a TSI 3475 condensation monodisperse aerosol generator (CMAG) (TSI, Shoreview, MN, USA). Its working principle relies on aerosolizing an aqueous salt solution in an atomizer and drying the obtained aerosol in a diffusion dryer, resulting in salt crystals of roughly 100-nm diameter. In a saturator, these salt nuclei are bubbled through heated aerosol material and the obtained nuclei-vapor mixture is subsequently heated in a re-heater unit, where complete evaporation of the aerosol material is assured. Cooling down in a condensation chimney causes super-saturation and homogeneous condensation of the vapor onto the salt nuclei. By varying the temperature in the saturator and the volume flow through the saturator, and by removing a part of the salt nuclei via filtration, the amount of aerosol material per salt nucleus, and thereby the resulting particle size, can be modulated.

For our application, we used a 0.5% aqueous solution of DSF, (Sigma-Aldrich, Munich, Germany) as the source of the salt nuclei, onto which glycerol (>99.5%, Sigma Aldrich) as aerosol material was condensed. The applied CMAG settings are listed in Table 1.

2.2. Aerosol characterization

Various aerosols of different particle number-size distributions could be generated in the CMAG, four of which were selected for more detailed characterization (Table 1).

In eight independent repetitions, particle number-size distributions and their modes, mean aerodynamic particle sizes and their geometric standard deviations (GSDs), and median aerodynamic particle sizes were measured continuously during 20–25 min, using a TSI 3321 Aerodynamic Particle Sizer (APS) with an accessory dilution unit (TSI 3302 A Aerosol Diluter, 100:1 capillary inserted). The APS was set to report data averaged over 10 s, and the obtained 120–150 data points per repetition were averaged for assessing intra- and inter-repetition stability of the aerosol generation.

Particle concentrations were also measured, but as the APS was operated at the upper limits of reliably measureable particle densities, the reported values are expected to underestimate the actual concentrations. Since in addition, the aerosol density was primarily captured by the DSF and aerosol mass flow rates, particle concentrations reported by the APS are not further used as quantitative data.

To derive aerosol mass deliveries in an aerosol exposure system based on fluorometric quantification of DSF delivery, the DSF and aerosol mass flow rates through the system during test exposures need to be determined. This was achieved by trapping the aerosols on weighted Cambridge filter pads (CFPs) (Borgwaldt KC, Hamburg, Germany) for defined periods of time (1–5 min, depending on the aerosol size) followed by determination of the deposited aerosol mass and elution and fluorometric quantification of the deposited DSF. To characterize the four selected aerosols, this was done with the APS, but not the VC24/48 being installed downstream of the CFP-holder. Trapping was performed during the APS measurements (*i.e.* APS measurements were interrupted), the APS data therefore describe the same aerosol flows as the CFP data. The aerosols trapped on CFPs were eluted from the filters by incubation in 10 mL Dulbecco's phosphate-buffered saline

Please cite this article as: Steiner, S., et al., A new fluorescence-based method for characterizing *in vitro* aerosol exposure systems, Toxicology in Vitro (2016), http://dx.doi.org/10.1016/j.tiv.2016.09.018