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Key challenges for in vitro testing of tobacco products for regulatory applications: Recommendations for dosimetry

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Abstract

The Institute for In Vitro Sciences (IIVS) is sponsoring a series of workshops to develop recommendations for optimal scientific and technical approaches for conducting in vitro assays to assess potential toxicity within and across tobacco and various next-generation products (NGPs) including heated tobacco products (HTPs) and electronic nicotine delivery systems (ENDSs). This publication was developed by a working group of the workshop members in conjunction with the sixth workshop in that series entitled "Dosimetry for conducting *in vitro* evaluations" and focuses on aerosol dosimetry for aerosol exposure to combustible cigarettes, HTP, and ENDS aerosolized tobacco products and summarizes the key challenges as well as documenting areas for future research.

KEYWORDS
aerosol, dosimetry, next-generation products (NGPs), smoke

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1 | INTRODUCTION

1.1 | Background

Since 2018, the Institute for In Vitro Sciences (IIVS) has been conducting a "Workshop Series to Identify, Discuss and Develop Recommendations for the Optimal Generation and Use of *In Vitro* Assay Data for Tobacco Product Regulation." The sixth workshop in that series—"Dosimetry for conducting *in vitro* evaluations"—was held on November 30 to December 1, 2021, and was attended by scientists from industry, government, contract research organizations, and academia. Prior to the workshop, a smaller working group (the authors of this manuscript) prepared background information on the current state of the art for dosimetry, which included potential conclusions and recommendations and also identified issues needing future research. This information was presented and discussed during the workshop. An overview of the workshop along with final conclusions and recommendations agreed upon by the workshop participants will be published.

1.2 | Why is dosimetry important?

The health effects of substances exposed to humans via the inhalation route are of considerable interest to many people and have led to the development and use of in vitro models of the respiratory tract. The inhalation route of administration for compounds has several advantages such as a rapid onset of action for drugs and chemicals, and it is predominantly noninvasive. This route of exposure comprises the extra thoracic airways including the nasal and oral cavities, the larynx, trachea, and lungs. The lungs have both a high permeability to compounds due to a large blood supply and large absorptive surface area. Critically, the exposure to compounds via the lungs avoids first-pass metabolism in the liver, which is important for compounds removed by metabolism.[1] The major challenge, however, in inhalation toxicity is the appropriate and accurate determination of the delivered dose (dosimetry). The effective delivered dose (the percentage of the inhaled dose that enters the bloodstream) is key; however, this is not easy to calculate due to numerous factors that can affect lung deposition, for example, particle size, anatomy of the respiratory tract, breathing patterns, and aerosol type (solid or liquid particles, gas phase, or combination thereof).[2,3] After deposition in the lung, some particles dissolve and absorb into the systemic/pulmonary circulation, whereas others are cleared from the lung by pulmonary metabolism, alveolar macrophages, and higher up the respiratory tract by mucociliary clearance (MCC). Similarly, substances forming liquid aerosols may change their phase by evaporation processes or become absorbed from the gas phase into the liquid phase, thereby modulating the delivered dose.[3]

Understanding dosimetry in terms of delivered dose is essential for placing scientific data gained from in vitro human models and in vivo animal models into context with human exposure. In terms of in vivo modeling of dosimetry, delivered doses can be measured/estimated by a series of methods including the use of particle size parameters such as particle number, size, and mass and software to estimate/predict lung deposition (e.g., using multiple pathway particle dosimetry [MPPD] model software). Dose may also be measured directly on the cell surface area in the in vitro environment by using methods such as quartz crystal microbalances (QCMs),[4] glass plates to measure deposited concentrations,[5] or predicted means such as computational fluid dynamics (CFD).[3]

Data generated from in vitro studies conducted preferably at the air–liquid interface (ALI) or in vivo inhalation studies are preferable for extrapolation to derived human doses. However, this can also be addressed using traditional standard toxicological risk assessments with the use of safety factors to quantify the probability and potential impact of a risk, which is an approach used by many regulatory agencies for decision-making purposes.[6,7]

Human dosimetry derived from in vitro data may also be accomplished by using in vitro to in vivo extrapolation (IVIVE), extrapolating effect concentrations seen in vitro to the corresponding human blood concentrations using a combination of both dosimetry and physiologically based pharmacokinetic (PBPK) modeling.[8,9] An essential step in this IVIVE process is the identification of relevant in vitro assays for specific in vivo endpoints where the mode of action is known. It should be noted that the validity of these methods relies heavily on the accuracy of the in vitro response measurements and how relevant the in vitro assay is to the in vivo mode of action. Indeed, the biological relevance of the in vitro assays to in vivo endpoints impacts the ability of the IVIVE approach to provide an accurate estimation of an equivalent administered dose (EAD), that is, the estimated external dose in humans that may induce similar bioactivity reflected by the in vitro assay.[10–12]

These methods that place in vitro results in an in vivo context are gaining increasing importance in scientific decision making[13] and in the context of NGP evaluations. Chang et al.[11] and Zhang et al.[12] used an IVIVE approach to assess the toxicity of ingredients and their mixtures in e-cigarette aerosols. The authors concluded that "IVIVE is a useful tool for interpreting *in vitro* data in the context of *in vivo* human exposure and can be applied to mixtures assessment for hypothesis generation and preliminary risk assessment."[12] This underscores the necessity to conduct appropriate and, relevant, in vitro studies utilizing the most appropriate dosimetry measurements to facilitate subsequent data use in applications such as IVIVE extrapolations.

1.3 | Where should dosimetry be applied (smoke/aerosol from the sample to exposure to cells)?

The exact application of dosimetry measurements largely depends on the exposure system being used but should be based on sampling the deposition and interaction of particles and gases or vapors at the cell surface. The determination of dosimetry in an ideal world is the measure of the internal dose or even concentration at the molecular target (biologically effective dose)[14–16] within the cells for the chemicals of interest. However, the difficulty in directly measuring cellular dose in

submerged cultures poses a significant obstacle to application of target tissue dosimetry for in vitro systems.

For example, assessment of nanoparticle and microparticle toxicity is challenging due to agglomeration of nanoparticles in liquids.[17] As a consequence of this, the target tissue paradigm for dosimetry and hazard assessment for nanoparticles has largely been ignored in favor of using alternative indirect methods of potential exposure such as μg particle/ml culture medium, particle surface area/ml, or particle number/ml.[17]

The nominal concentration measured in the cell culture medium of in vitro studies can differ significantly from the cells' internal dose, as different nanoparticle characteristics may affect the ability of particles to reach the cells at the bottom of the culture dish.[18] Due to the difficulties in extrapolating the concentrations of particles in submerged cultures to the concentrations of particle deposited/available at the cell surface and then internalized, the preferred exposure route is to minimize surface liquid and use cells exposed at the ALI if at all possible. Particle properties including particle size, surface charge, transformation, solubility, or agglomeration state and chemical properties are altered when particles are exposed in solution (submerged cultures) and are also dependent on the specific composition of the cell culture medium.[19,20] For example, test chemical hydrophobicity is known to affect its distribution and bioavailability in vitro, rendering only a small fraction available in the aqueous phase for cellular uptake.[21] Chemicals may bind to serum within the medium, may be absorbed into the cell culture setup plastic, or may volatilize to varying degrees dependent on chemicals' properties.[16,21,22] The chemical solvent used, for example, DMSO, may also have an influence on the way chemicals are distributed and on their availability to cells; however, the exact mechanism is unknown.[21]

Dosimetry determination is also key for tissues exposed at the ALI to accurately determine the dose deposited on to the cell surface and the dose ultimately available to be absorbed by the cells. The more physiologically relevant in vivo-like in vitro models cultured at the ALI is therefore becoming a realistic and efficient tool for lung toxicity testing.[23] The ALI is the preferred exposure route following exposure to either aerosolized or gaseous form of air pollutants, including cigarette smoke (CS) due to the improved physiological relevance of this exposure route for humans when using human 3D reconstructed tissues (e.g., several studies[23–25]). Cao et al.[26] also highlighted the value of utilizing the ALI for the evaluation of cigarette and NGP (e-cig) aerosols and emphasized the suitability of these in vitro methods for the evaluation of pathological changes induced by inhaled substances as well as indicating the possibility to use these systems to conduct subacute exposure studies with e-cig aerosols. The author concluded that "although the *in vitro* toxicity assessment of e-cig aerosols is still in its infancy, the primary cell–based ALI airway tissue model system has proven to be a relevant platform for screening alternative tobacco products as well as elucidating the mechanisms underpinning e-cig aerosol toxicity to human bronchial epithelium."[26]

As these NGP products develop and the technologies evolve, ALI testing will continue. To maximize the use of in vitro toxicological data generated in this area, however, a means to compare information generated using different exposure systems is required. More recently, Bishop et al.[27] presented a means by which data generated using different aerosol generation systems could be compared. The authors documented that cellular responses to generated e-cig aerosols were comparable when applying an undiluted protocol. This research is especially vital as in vitro methods are critical in the evaluation and screening of NGP, and as such, adopting a methodology that facilitates comparison between data utilizing different aerosol-generating systems provides a tangible means to "bridge" information, which is essential to maximize the use of in vitro data to aid in the screening and or evaluation of NGPs.[27]

1.4 | The necessity to understand what changes occur in the sample composition between smoke/aerosol generation and cell exposure at the ALI

CS is a complex and dynamic aerosol consisting of both solid and liquid particles, called the particulate phase (able to be trapped on a filter pad), suspended in a mixture of vapors and carrier gases, the gas phase (commonly referred to as the gas–vapor phase (GVP)) passes through a filter pad.[28] CS is generated by several complex and overlapping processes consisting of combustion, pyrolysis, pyrosynthesis, distillation, sublimation, and condensation.[29] The small highly reactive compounds formed in the CS gas phase are generated by pyrosynthesis (including carbonyls and NO[30]). Because combustion takes place during smoking, a substantial number of solid particles are released from the combustion of the tobacco itself and transferred to the mainstream smoke. This production of solid particles is not seen with next-generation products (NGPs) that heat and do not combust a tobacco rod or heat an e-liquid, for example,[31,32] giving rise to far less chemically complex aerosols.[33-35] Pyrolysis is not present in NGPs, but reaction and degradation products are generated when an e-liquid is heated[34] due to the degradation of the heated constituents of e-liquids including propylene glycol, vegetable glycerin, and some flavors.[36]

Once the CS or aerosols are formed, compounds can rapidly react with other constituents to form additional compounds, this is known as aging; that is to say the smoke composition can change with time after generation (temporally dynamic).[28] Hence, one of the challenges in using CS exposure systems is getting samples as quickly as possible to the cell exposure chambers without excessive deposition of solid and liquid particles on the walls of the equipment. For this reason, tubing connecting the generation of sample and the exposure apparatus are kept as short as reasonably possible. For a comprehensive review on the history of methods and devices for generation, exposure, and collection, please see Klus et al.[28]

Exposure systems consist of three parts:

1. a smoke- or aerosol-generating component,
2. means of connection/delivery for the diluted or undiluted smoke or aerosol, and
3. cell exposure system.

Once the aerosol is generated, typically using a smoking robot programmed with a fixed puffing regime (puff duration, puff volume, and time between puffs), CS or aerosol is usually delivered over a fixed time via a piston/syringe system. The fixed puffing regime is one of several means enabling results to be compared across other studies, along with other determinants described below. Dilution of the smoke/aerosol is possible as it transfers through the system. It is at this stage, post-syringe, that these exposure systems start to diverge in design. The type of tubing, diameter and length of tubing, dilution principle (use of pistons and vacuum rate from an airstream), losses on the walls of connecting pipe work, and subsampling of the aerosol significantly affect the delivered dose to the cells in the exposure chamber.[28,37] The final aspect, which all systems share, is the exposure chamber/module, where cells are housed and exposed with the cells raised up from the cell media, the basal surface in contact with the cell media, and the apical surface being exposed to CS/aerosols.

The physical and chemical behavior of CS, heated tobacco product (HTP), or electronic nicotine delivery system (ENDS) aerosols are highly variable and can differ both temporally (highly reactive constituents can react in seconds) and in terms of composition due to a tendency for disparate deposition of particles and vapor and condensation as the CS/aerosols cool down on the exposure system walls and connecting tubes. For this reason, the design and construction of aerosol generation and exposure chamber systems are likely to alter an aerosol's characteristics (e.g., its particle size distribution [PSD] or the partitioning of its constituents in the solid, liquid, and gas phases). To achieve meaningful results when testing aerosols, a key requirement is to understand and potentially control the processes influencing the aerosol delivery, including aerosol aging by getting the samples to the cells as quickly as possible and measuring deposition at various points in the system. These requirements apply to analytical characterizations as well as to in vitro and in vivo testing.[31,32]

Acronyms, synonyms, and abbreviations used throughout this manuscript are shown in Table 1.

2 | NEED TO UNDERSTAND THE PROPERTIES OF THE EQUIPMENT AND TO CHARACTERIZE THE SAMPLE

Cells may be experimentally exposed to CS in a variety of ways including trapped fractions in several defined media, such as bubbled phosphate-buffered saline (PBS), cell media, ethanol, and DMSO, or using a glass filter and aqueous extract combined. For an overview of the wide variety of trapping methods used, see Smart and Phillips[38] and Klus et al.[28] Trapping of CS/aerosols in impingers can be difficult to extrapolate across studies due to the differences in methods used and exact impinger setup.[38] Impingers are used to trap CS/aerosols to prepare liquid cigarette whole-smoke samples. The different fractions captured depend on the media used in the impinger and partly on the volume of media used due to possible saturation. These fractions have then been used to measure trapped constituents such as carbonyls and nicotine. Considering this information, exposing cells at the ALI exposes the cells to whole CS aerosol and is the most relevant to the human exposure scenario for inhaled products and looking at direct effects on the lungs.[28,39,40]

With the ALI exposure of cells, dosimetry is typically performed using blank wells with round glass slides covering the cell growth area or full of PBS or cell media and quantified for the analytes of interest; quartz microbalances have been typically used for cigarettes.[5,41–48] Traditionally, nicotine has been the measured analyte of choice due to its high stability, ease of measurement, and its relevance to smokers and NGP users[49,50]; for further details, please see Table 2.

3 | THE GENERAL CHARACTERIZATION OF SMOKE/AEROSOLS GENERATED USING DIFFERENT MACHINES AND METHODS

As previously detailed, CS, HTP, or ENDS aerosols can be collected and processed to produce extracts suitable for submerged culture toxicology assessments. To produce these extracts, CS or aerosol is typically collected using a glass fiber filter connected in series with a liquid-filled impinger. Particulate-phase material is collected on the filter, and GVP compounds are collected in the impinger. Extracts are prepared by extracting the filter in a suitable solvent, with DMSO being the most commonly used. GVP compounds are typically collected in a chilled impinger containing PBS solution. The use of aqueous media in the impinger results in the collection of primarily water-soluble GVP compounds. Extracts have been prepared using a range of smoking and puffing machines including 20-port rotary machines[68] and linear smoking machines including both single-port[69] and multi-port linear smoking machines.[70] CS extracts are typically collected under ISO 3308[71] or ISO 20778,[72] and ENDS product extracts are typically collected under ISO 20768.[73] Extracts from other products, including cigars or waterpipe, have been collected under suitable smoking regimes.[74–77] More recently, waterpipe tobacco has been smoked using the standardized ISO 22486[78] conditions.[79]

A wide range of smoking or puffing machines have been used to generate whole smoke or aerosol for in vitro exposure systems. The most common approach includes the use of smoking machines capable of the continuous delivery of smoke or aerosol to the in vitro exposure system. Examples of commercially available continuous delivery systems include the 5-port Burghart MSB-01 (Burghart, Wedel, Germany),[80] the 5-port SAEIVS (Burghart, Wedel, Germany)[81], the 8-port Borgwaldt RM20S (Borgwaldt KC, Hamburg, Germany),[60] the 10-port VITROCELL® VC 10 (VITROCELL® Systems, Waldkirch, Germany),[55] and the 30-port SM2000 (Philip Morris International, Neuchâtel, Switzerland).[46] These systems operate by using a series of syringes to both puff and deliver the smoke or aerosol to the in vitro exposure system. A uniform delivery of the smoke or aerosol to the in vitro exposure system is maintained by controlling the exhaust timing of the primary syringe or through the use of a secondary syringe

TABLE 1 Terminology, synonyms, and abbreviations

Terminology[a]	Synonym(s)[b]	Abbreviation[a]	Description[c]
Air–liquid interface		ALI	Cells housed at an interface that facilitates basolateral feeding and apical aerosol exposure
Biologically effective dose	Metabolic dose	N/A	Dose delivered to the specific intracellular process that results in the primary biological response
Cigarette smoke	- Tobacco smoke - Combustible cigarette smoke	CS	N/A
Cambridge filter pad	N/A	CFP	A filter pad used to capture particulate material
Delivered dose	Cellular dose	N/A	Dose delivered to the surface of the cells that results in the biological response
Dosimetry	- Delivered dose - Biologically effective dose	N/A	Quantification of delivered dose. Often related to the delivered dose to the target tissue at the exposure interface. Can be used to describe chemical and physical approaches to measure/characterize test articles prior to exposure
ENDS	- Electronic nicotine delivery system - Electronic cigarette or e-cigarette - Electronic vapor product (EVP) or e-vapor product	e-cig ENDS	An electronic system that aerosolizes an e-liquid
Exposure interface	N/A	N/A	The interface at which the cells are exposed
Electronic vapor product	- Electronic cigarette - ENDS	EVP	An electronic system that aerosolizes an e-liquid
Gas–vapor phase	Vapor phase	GVP	Normally referred to as the aerosol phase that is not collected using particulate-trapping techniques such as Cambridge filter pads (CFPs)
Quartz crystal microbalances	N/A	QCMs	A quartz crystal disc used to gravimetrically measure deposited mass based on changes in oscillating frequencies
Heated tobacco products	- Tobacco heating products (THPs) - Heat not burn (HnB) -Tobacco heating product (THP) - Tobacco heating system (THS)	HTPs	A tobacco product that heats the tobacco to create an aerosol that the user inhales
Internal dose	Intracellular dose	N/A	Dose present within the cells that results in the biological response
Next-generation nicotine and tobacco products	N/A	NGPs	Umbrella term for HTP, ENDS categories, and other oral products such as modern oral tobaccos, snus, and nicotine inhalers
Smoke/aerosol exposure chamber	- Smoke/aerosol exposure module - Module - Chamber	N/A	The apparatus in which the cells are housed at the ALI and introduced to the exposure aerosol
Smoke/aerosol exposure system	- Smoking robot - Whole-smoke exposure system - Smoke exposure platform - In vitro aerosol system	N/A	The complete smoke or aerosol exposure system including the smoking head, associated dilution system, and the exposure chamber
Total particulate matter	- Particulate matter - Particulate material	TPM	The total particulate material captured on a Cambridge filter pad and eluted for testing using traditionally DMSO as a solvent (but is not exclusive to DMSO)
Whole aerosol	- Cigarette smoke aerosol - HTP (whole HTP aerosol) - ENDS (whole ENDS aerosol)	N/A	Refers to the generation of a "complete" smoke/aerosol through in vitro aerosol exposure systems. Umbrella term for freshly machine-generated aerosols from

(Continues)

TABLE 1 (Continued)

Terminology[a]	Synonym(s)[b]	Abbreviation[a]	Description[c]
			HTP (whole HTP aerosol) and ENDS (whole ENDS aerosol)
Whole-aerosol extracts	N/A	N/A	The capturing of smoke or aerosol constituents in an aqueous medium (e.g., either cell culture media or PBS)

[a]Terminology and abbreviation used in this manuscript.
[b]Not all synonyms are used in this manuscript, but they are used within the wider research environment.
[c]Description based on working group and current opinion.

system. Single-port smoking machines have also been used to generate smoke or aerosol on a puff-by-puff delivery of smoke or aerosol to in vitro exposure systems. Examples of commercially available puff-by-puff delivery systems include the Borgwaldt RM1/LM1[82] and the VITROCELL VC 01.[83] CS is typically generated under ISO 3308 or ISO 20778, whereas ENDSs are typically generated under ISO 20768.[73] Smoke or aerosol from other products, such as waterpipes, has been generated using suitable smoking regime conditions.[79,84] An overview is presented in Table 3.

4 | CHARACTERIZATION OF DIFFERENT SAMPLE TYPES

4.1 | Pad collected/condensates

CS, HTP, or ENDS aerosols for in vitro toxicological assessment are typically generated on either linear or rotary smoking machines, depending upon the requirements of the study.[86]

Various methods to trap the aerosol, or fractions of aerosol, exist including glass fiber filter pads used to collect the particulate-phase fraction that is subsequently extracted with DMSO or an alternative suitable solvent to a specific concentration.[87–94] Alternatively, impaction trapping may be used for collection. This method forces high-pressure aerosol through a capillary or nozzle and captures aerosol fractions via inertial impaction, which may be subsequently collected onto a suitable surface.[28]

Electrostatic trapping of the "whole" aerosol is where a positive electrode is in the center of a glass tube where the aerosol enters. The tube is surrounded by a negative electrode made of stainless steel, and an electric field is created to attract the charged aerosol particles to the inner glass tube, which collects the aerosol that passes through, and these are subsequently eluted with DMSO or another appropriate solvent.[31,86,94–96] Electrostatic precipitation can be used with almost any product type where an aerosol is generated. An alternative method uses a cold trap that has a glass impinger cooled and held on dry ice and acetone or methanol or a mixture of the two, to maintain the environment at −78°C. When the aerosol enters the impinger, the low temperature causes the aerosol to condense on the sides of the apparatus, which can then be eluted with an appropriate vehicle, for example, DMSO.[28,31]

The TPM collected on glass fiber or similar filter pads, and indeed the condensates collected in cold traps and electrostatic precipitators, can be chemically characterized to ensure that consistent test articles have been generated.[94] For pad-collected matter, this usually involves the determination of the weight of particulate matter collected and the determination of nicotine,[97] water,[98,99] and humectants, as well as other constituents that may be of interest. The use of nicotine and TPM weights can also be used as quality control checks for consistency of the test article generated. Furthermore, the measurement of constituents in frozen vehicle extracts can have nicotine determined to demonstrate stability of the extracts.

There are essential differences between the different collection methods. For example, pad-collected aerosol will predominantly collect the particulates in the aerosol and not capture volatile constituents. Aerosols that are captured in cold traps, electrostatically, or via an impaction trap are more likely to contain the particulates, some semi-volatile compounds, and possibly some volatile compounds.

4.2 | Samples trapped in liquid media

CS or aerosol generated from NGPs can be bubbled through a liquid solvent to generate a test article for in vitro assessment. There are two main ways of generating the test article: The first is assessment of the GVP where a glass fiber filter pad is placed prior to the collection so that the particulate matter does not interact with the liquid solvent, and the second method is to generate an aqueous extract where both phases of the smoke or aerosol interact with the solvent.

Sample generation, smoking regimens, and collection methods for cigarettes are generally performed as per ISO 3308[85] or ISO 20778.[72] Methods for NGPs are generally addressed using ISO 20768[73] or a modified version of ISO 20778. ISO/AWI 5501 is currently drafting HTP puffing regimes, but this activity remains ongoing at the time of publication.

A standardized method is available for the generation of GVP samples as defined in the Canadian health authority (Health Canada) Tobacco Reporting Regulations (TRR) Method (T-502 2017).[100] In summary, the smoke or aerosol is passed through the glass fiber filter before being bubbled through an ice-cold liquid solvent. The most common solvent used is PBS.[70,101,102] It is important that the PBS is ice-cold to ensure that the volatile compounds within the vapor phase

TABLE 2 Reported online and offline ALI dosimetry parameters for cigarette smoke and e-vapor aerosol

Online dosimetry parameters			
Parameter	**Where measured**	**Method**	**Advantages/limitations**
Mass deposited	Surface of exposure well with no cells	Quartz crystal microbalance[41–48,51] Predictions via computational fluid dynamics (CFD)[3,52]	Quick continuous measurements; excellent detection limit Not suitable for all aerosols including ENDS
Carbon monoxide (CO) concentration	Exit of system	CO monitor[53,54]	Quick; measurement frequency selectable Chemical specific; not applicable to particle phase
Aerosol mass	Sample of exposure atmosphere or in dilution system (bar)	Photometers/optical particle counters[45,51,53,55]	Quick; continuous measurement Limited placement within system; must be calibrated to aerosol mass for each dilution
Nicotine, glycerol, propylene glycol, and water	Various ports in high-throughput ALI exposure system	FTIR[31,56] SPI-MS[57]	Chemical specific; excellent detection limits Specialized equipment and skilled operator required
Various chemicals	Various ports in high-throughput ALI exposure system	PI-TOFMS[31,32]	Chemical specific; excellent detection limits Specialized equipment and skilled operator required
Particle size	Various ports in high-throughput ALI exposure system	Aerodynamic particle sizer (TSI 3321)[57–59] Scanning mobility particle sizer (SMPS) (TSI 3080)[44]	Online monitoring Aerosol is diluted 100-fold (may introduce artifacts)

Offline dosimetry parameters			
Parameter	**Where measured**	**Method**	**Advantages/limitations**
Mass deposited	Surface of exposure well with no cells	Gravimetric[60,61] Glass plates to measure deposited concentrations[5]	Direct; relatively quick Does not capture gas-phase constituents
Number of particles	Surface of exposure well with no cells	Fluorescence[44,45,54,58–60,62]	Quick; relatively easy Fluorescence must be calibrated to mass; sensitivity depends on equipment and fluorescent marker
Solanesol and acetaldehyde	Surface of exposure well with no cells, membrane removed	Analytical chemistry HPLC[63]	Specific; markers of particle- and gas-phase constituents Analytical method must be developed for collection matrix
Mass of nicotine	Exhaust line and cell culture media from reservoir	Analytical chemistry[46,49,64,65]	Specific; can capture gas and particle phase Analytical method must be developed for collection matrix
Carbonyls	In-line impingers and cell culture media from reservoir	Analytical chemistry[46,66,67]	Specific; captures gas phase Analytical method must be developed for collection matrix
Distribution of MS among exposure inserts	Surface of exposure well with no cells	WST-1 reduction[46]	Quick; relatively easy Not chemical specific, limited dynamic range, fluorescence must be calibrated to mass, and sensitivity depends on equipment

Abbreviations: ALI, air–liquid interface; ENDS, electronic nicotine delivery system; FTIR, Fourier-Transform Infrared spectroscopy; HPLC, high-performance liquid chromatography; MS, mass spectrometry; PI-TOFMS, photo-ionization time-of-flight mass spectrometer; SMPS, scanning mobility particle sizer; SPI-MS, single-photon ionization mass spectrometry.

are retained. Health Canada T-502 recommends the GVP to be applied to the test system within an hour of generation due to potential loss of captured volatile and semi-volatile constituents. Analysis of the glass fiber filter can be undertaken as described in ISO 4387[103]; the weight of particulate matter can be used to assign an equivalent total particulate matter (mg TPM equivalent/ml). Additionally, chemical analysis of the GVP-bubbled solvent can be performed to quantify captured constituents.

TABLE 3 Overview of ISO smoking regime parameters

Regimen	Puff volume (ml)	Puff duration (s)	Puff interval (s)	Reference
ISO 3308	35	2	60	ISO 3308:2012 Routine analytical cigarette-smoking machine—definitions and standard conditions[85]
ISO 20778	55	2	30	ISO 20778:2018 Cigarettes—routine analytical cigarette smoking machine—definitions and standard conditions with an intense smoking regime[72]
ISO 20768	55	3	30	ISO 20768:2018 Vapour products—routine analytical vaping machine—definitions and standard conditions[73]
ISO 22486	530	2.6	20	ISO 22486:2019 Water pipe tobacco smoking machine—definitions and standard conditions[78]

A standardized method is not available for the generation of whole-aerosol aqueous extracts; however, the procedure would be as described above for GVP but without the presence of the glass fiber filter. Aqueous extracts are generally produced in a wide range of cell culture medium suitable to the in vitro assay.[104–106] Aerosol exposure has also been performed with a test system such as the Ames assay where the bacterial inoculum is suspended in PBS and bubbled with whole smoke or aerosol.[82] Due to the lack of a glass fiber filter and the subsequent inability to measure weight or w/v of TPM to quantify the exposure dose, nicotine analysis of the extract is performed to enable characterization.

As with any of the methods described for generation and testing of sample types derived from aerosolized tobacco products, it is important to characterize and understand the trapping system that is being used for both GVP and whole-aerosol aqueous extracts. There is a wide selection of impingers and bubblers available with differing amounts of headspace. Consideration should be given to interaction of smoke or aerosol with the solvent and if a frit or glass beads should be employed to increase the bubbling and therefore increase the surface area for gas transfer to the solvent. It is possible to connect multiple impingers in series, and subsequent chemical analysis can demonstrate capture efficiency. A wide range of commercially available smoking machines have been used, both linear and rotary. There is no consensus as to the type of smoking machine to use, but the smoking machine should be assessed and characterized alongside the impinger system for compatibility, total leakage, and trapping efficiency.

4.3 | Whole-aerosol methods

In vitro systems exposing 2D cultures or 3D tissue constructs exposed to freshly generated whole aerosol at the ALI add a level of complexity to the overall exposure system construction and determination of the delivered dose to the cells.[107,108] The exposure system will include some form of smoking or vaping machine capable of generating a representative aerosol from the combustible or NGPs to be tested. There are a wide range of aerosol-generating machines that are commercially available or developed by individual laboratories to meet their specific needs. Demonstration of consistent aerosol generation from reference or representative tobacco products is a first important step in understanding the entire exposure system. Thorne et al.[37] demonstrated comparable aerosol generation, based on nicotine, from nine in vitro whole-aerosol exposure setups representing seven different systems, across five geographical locations, using a Kentucky Reference 3R4F combustible cigarette when systems were well maintained and run under standard atmospheric and puffing conditions.

Once the representative aerosol is generated, it must be delivered to the exposure portion of the system. Aerosol can be delivered undiluted or undergo dilution prior to delivery to the exposure module(s). Several systems perform aerosol dilution by mixing the aerosol with clean air, either within a piston or with a constant controlled flow of air.[28,109] Aerosol dilution allows exposures to be conducted over a range of delivered doses over a set time, whereas undiluted aerosol can be delivered on the basis of time or puff number to alter the amount of aerosol exposed to the cell cultures.[110,111] How the aerosol is delivered and deposited into the exposure module is another crucial step in understanding the performance of the system. There are several published studies examining aerosol or particulate deliveries into a host of exposure modules to determine deposition consistency or patterns on cell culture surfaces.[46,52,63,110,112–115]

Determination of the delivered dose to the cells is essential in understanding the aerosol's impact and relevance to the biological responses measured and also important in facilitating any possible comparisons between laboratories and exposure systems. Examples of early dosimetry approaches included using percent (%) cigarette or smoke,[46,116,117] dilution airflow rate,[118,119] or number of cigarettes.[120] Implementation of real-time dose determinations included the use of QCMs to measure the mass of deposited particulate material,[51,121] laser photometers to monitor aerosol being delivered into the exposure module,[45] or online chemical analysis.[31,32] These methods have their strengths and limitations; for an overview, see Table 2.

The use of chemistry methods to quantify aerosol constituents captured or delivered is also being used to determine the amount of aerosol delivered. Nicotine, an obvious representative compound, has been widely used across the spectrum of tobacco- and nicotine-containing products[49] to measure delivered dose. Other constituents have also been utilized, including those found in either the particulate

or gas phases of the aerosol.[31,32,46,63] To efficiently use quantitative measures of aerosol constituents, researchers need to characterize the levels of those compounds generated by the product prior to performing whole-aerosol exposures. A recent publication[122] demonstrated the variability of methods and approaches laboratories utilize for whole-aerosol in vitro cytotoxicity. Harmonization of methods and the use of quantitative measures of aerosol constituents when exposures are performed under standard puffing and laboratory conditions, however, should allow comparisons between laboratories and different whole-aerosol exposure systems.[123]

5 | CHARACTERIZATION OF DELIVERED DOSE TO CELLS AND ANALYTICAL CHEMISTRY FOR DOSIMETRY

As indicated previously, aerosol dosimetry (delivered dose), understanding what is applied or delivered to cells and to what they are exposed, is critical to understanding the biological responses.

Although the chemical complexity and temporal dynamics of tobacco smoke and NGP aerosol currently preclude determining the internal and biologically effective dose, estimating or providing the delivered dose has been done using various online (during experiment) and offline methods (analyzed after the experiment) in ALI in vitro studies (Table 3).

Sauerbrey[124] developed the QCM and had three criteria for valid measurements: (1) Deposited mass must be small compared with mass of quartz crystal; (2) deposited mass is rigid; and (3) mass is evenly distributed over the crystal surface. Although the QCM technique works for tobacco smoke, some investigators allowed water and potentially volatiles to evaporate prior to a final measurement, which may affect measurement accuracy.[125] Given the criteria noted above, the QCM technique would not be expected to work well for ENDS aerosols as they are characterized by liquid droplets that are not rigid. Steiner et al.,[59] Adamson et al.,[49] and Keyser et al.[51] have recently confirmed the limitations of the QCM technique using ENDS aerosols. Also, uniformity of particle deposition across the surface of the cell inserts, and therefore the QCM, has recently been questioned in a couple of high-throughput ALI in vitro exposure systems.[114,115]

The use of optical particle counters, photometers, and specialized gas analyzers may be limited to where they can sample from within the ALI exposure system, and measurement at the cell surface is recommended. Measurement of specific chemicals online or offline from tobacco smoke or ENDS aerosol requires specialized equipment and collection techniques (use of PBS, DNPH, DMSO, etc.) that must be validated prior to use. When different dilutions are used, chemical amounts should ideally be determined at each dilution. Steiner et al.[67] reported that diluting tobacco smoke approximately 7-fold resulted in 3- to 30-fold decreases in chemical delivery to the cell surfaces, depending on the specific chemical. Another dosimetry concern with ENDS aerosols is osmolarity. Even use of gravimetric samples from a cell insert collected during an experiment can present challenges. If high humidity (>65–70%) is used during the experiment, then water and other chemical evaporation could occur after removal from the ALI exposure system and during weighing.

TABLE 4 Table of recommendations

Area	Recommendations
Methods	Methods used by laboratories to collect, trap, sample, and quantify the dosimetry parameter (delivered dose of aerosol constituents) should be thoroughly documented, standardized, and/or validated and be capable of adaptation to a variety of in vitro whole-aerosol exposure systems to allow comparisons across laboratories, other in vitro and ALI exposure systems, and in vivo and human studies.
Dosimetry parameter characterization	Dosimetry parameter characterization should be conducted in the ALI in vitro exposure system using experimental conditions (temperature, humidity, dilutions, etc.) and the same aerosol to demonstrate acceptable uniformity between all wells in the ALI in vitro exposure system. Dosimetry parameter characterization should include deposition efficiency, and if the aerosol is not a liquid droplet, then uniformity of deposition across the cell culture insert should also be confirmed quantitatively.
Dosimetry parameter(s)	Dosimetry parameters should be representative of the aerosol being assessed biologically in vitro. Additionally, the measured aerosol constituent(s) should represent the specific aerosol phase (particle or gas vapor phase) being studied, or in which phase the biological activity is thought, or found to reside. For example, a particle phase constituent should not be the only representative dosimetry parameter if the gas phase is shown to possess the biological activity being assessed in vitro. Hence, for CS, HTP, and ENDS aerosols, it is recommended that a minimum of two aerosol constituents representing the particulate and gas phases of the aerosol should be quantified for dosimetry purposes (e.g., nicotine and carbonyls in undiluted ENDS aerosols or glycerol and carbonyls in diluted ENDS aerosols). Dosimetry parameter(s) must be measured in a representative manner during each experimental run using chemical, physical, and in vitro methods (e.g., separate test on the test day and one of the replicate wells).

Abbreviations: ALI, air–liquid interface; CS, cigarette smoke; ENDS, electronic nicotine delivery system; HTP, heated tobacco product.

Aerosol dosimetry should not be exclusively represented by a dilution factor or percentage of delivered aerosol. Due to the considerable differences in aerosol compositions and deliveries among the range of nicotine delivery product types such as combustible cigarettes, HTP, and ENDS, utilizing a dilution or percentage approach will hinder or prevent any realistic and meaningful product comparisons. Currently, the most rigorous approach incorporates analytical measures, with standard and/or validated methods of particle- and gas-phase aerosol constituents to quantify the delivered dose. The aerosol dosimetry challenges of CS, HTPs, and ENDS aerosol also include the specific ALI system used for cell exposure. Therefore, a series of recommendations are proposed as outlined in Table 4.

6 | IDENTIFICATION OF ISSUES NEEDING ADDITIONAL RESEARCH

This publication was designed to focus on dosimetry, which is essential to understanding the biological response(s) in in vitro systems. It outlines the available methodologies, hardware, quantification methods, and challenges and highlights areas for additional research consideration, including the need for further research to better understand which aerosol constituents adequately represent each aerosol phase (particulate and gas) from NGP and reference cigarettes for specific biological endpoints.

Recommendations and areas for future research include that experienced, well-equipped laboratories should determine the aerosol constituents that best represent in vitro exposures in ALI systems and publish these results. All data should be accompanied with the justifications as to why the chosen aerosol constituents are adequately representative. Research is also needed to not only identify the most relevant aerosol constituents for each aerosol phase but also ensure that any analytical methods chosen for these aerosol constituents be as simple as possible with minimal associated cost. Collaborative studies are essential to facilitate interlaboratory comparisons of dosimetry measurements for standardization purposes.

Knowing the different hardware profiles for the different smoking or aerosol-generating machines, care should be taken to understand how these differences may impact the constituent profile after conducting puff profiles, and as such, the following should be considered: aerosol constituent losses along the exposure system, for example, is there a loss of constituents to tubing; understand/determine the run-to-run, day-to-day variability, and so forth for all ALI systems; and understand/determine smoke/aerosol aging in general and within each ALI system; specific studies should be designed to understand the various sources of total amount of dosimetric variability (e.g., product aerosol generation variability, in vitro exposure system variability, and analytical technique measurement variability).

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