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Lung Cell Exposure to Secondary Photochemical Aerosols Generated from OH Oxidation of Cyclic Siloxanes

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Abstract

To study the fate of cyclic volatile methyl siloxanes (cVMS) undergoing photooxidation in the environment and to assess the acute toxicity of inhaled secondary aerosols from cVMS, we used an oxidative flow reactor (OFR) to produce aerosols from oxidation of decamethylcyclopentasiloxane (D5). The aerosols produced from this process were characterized for size, shape, and chemical composition. We found that the OFR produced aerosols composed of silicon and oxygen, arranged in chain agglomerates, with primary particles of approximately 31 nm in diameter. Lung cells were exposed to the secondary organosilicon aerosols at estimated doses of 54–116 ng/cm² using a Vitrocell air-liquid interface system, and organic gases and ozone exposure was minimized through a series of denuders. Siloxane aerosols were not found to be highly toxic.

Keywords

oxidative flow reactor; personal care product; photooxidation; Vitrocell; air-liquid interface cell exposure; A549

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SUPPORTING INFORMATION

Details of individual experiments, system characterization, and additional information regarding SMPS data post-processing.

Declaration of interest

RJ Lee Group manufactures the thermophoretic sampler used in this study.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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1. INTRODUCTION

Over the past decade, research interest in siloxane and polysiloxane (silicone) compounds has increased markedly. While these compounds have been in-use since the 1940s, improved analyses coupled with increased interest in persistent anthropogenic chemicals has revealed widespread presence of organosiloxanes in the environment. Furthermore, the environmental degradation pathways that lead from parent compounds to the assumed stable oxidation product, silica (SiO_2), are complex and incompletely understood (Rucker and Kummerer, 2015). Chemical interconversion between organosiloxanes and discovery of both parent molecules and degradation intermediates in a variety of biological samples and environmental media has further stimulated research (Rucker and Kummerer, 2015; Mojsiewicz-Pienkowska and Krenczkowska, 2018).

Cyclic volatile methyl siloxane (cVMS) compounds are of particular interest due to their volatility and their wide use in personal care products and industrial applications (Wang et al., 2013; Rucker and Kummerer, 2015; Mojsiewicz-Pienkowska and Krenczkowska, 2018). cVMS compounds, most commonly decamethylcyclopentasiloxane (D5), are used ubiquitously as emollients in antiperspirants and in hair and skin care products because of desirable properties. While estimates of cVMS compound emissions vary, a frequently used air emission estimate for US D5 is 135 mg/person/day (Horii and Kannan, 2008; Navea et al., 2011; Janecek et al., 2017). Further, recent literature indicates that cVMS are non-negligible sources of VOC emissions in urban environments (McDonald et al., 2018) and that cVMS are dominant pollutants (~1/3 of volatile carbon) in classroom air (Tang et al., 2015).

The safety of the parent cVMS compounds has been extensively evaluated by both US and European oversight groups, with varying conclusions. Most regulatory jurisdictions have concluded that parent cVMS compounds pose a minimal health risk to humans and animals under typical use and concentration scenarios, although some have taken more conservative approaches and listed cVMS compounds for additional monitoring, evaluation, or phase-out (SCCS, 2010; Johnson et al., 2011; Rucker and Kummerer, 2015). These compounds generally evaporate quickly after application and thus are minimally absorbed. Human uptake of D5, typically by inhalation results in measurable bloodstream concentrations. The blood plasma half-life of D5 is approximately 2–3 days, and the compound is removed via partitioning into fat, exhalation from the airspaces, and metabolism into silanols (Reddy et al., 2008; Xu et al., 2012). Further studies of D5 and similar cVMS compounds have generally found little to no toxicity (Rowe et al., 1948; Johnson et al., 2011), with some exceptions observed for derivatives of the parent compounds (Albanus et al., 1975; Wang et al., 2013; Mojsiewicz-Pienkowska and Krenczkowska, 2018) and some effects seen in high concentration studies with animals (Rucker and Kummerer, 2015).

A large fraction of the cVMS are emitted to indoor and outdoor air after application due to their volatility, and thus become available for reaction in the atmosphere. cVMS react in the atmosphere with hydroxyl radicals (OH), generating a variety of products including non-volatile or semi-volatile compounds (Atkinson, 1991; Sommerlade et al., 1993; Wu and Johnston, 2016; Wu and Johnston, 2017). Recent work has established that siloxanes are

widely distributed, with the highest concentrations in urban population centers and more diffuse concentrations in surrounding areas (McLachlan et al., 2010; Genualdi et al., 2011; Xu and Wania, 2013; Yucuis et al., 2013; Bzdek et al., 2014; Janecek et al., 2017). The primary removal mechanism for siloxanes in the environment is predicted to be via photooxidation with OH radicals (Atkinson, 1991; Xiao et al., 2015); the OH reaction kinetics result in a long lifetime for D5 in the troposphere of approximately 10 days (Atkinson, 1991). Chamber-based experiments have demonstrated that these non-volatile and semi-volatile cVMS oxidation products can nucleate or condense onto preexisting aerosol particles via the reaction $\text{cVMS} + \text{OH} \rightarrow \text{o-cVMS (oxidized product)} \rightarrow \text{particle species}$ (Latimer et al., 1998; Chandramouli and Kamens, 2001; Wu and Johnston, 2016; Wu and Johnston, 2017; Janecek et al., 2019). Field measurements of the elemental composition of ambient aerosols further suggest that cVMS photooxidation may be a source (Bzdek et al., 2014). Further work demonstrated that the chemical conversion and incorporation of cVMS into aerosols modifies the transport and accumulation of cVMS in the environment (Latimer et al., 1998; Whelan et al., 2004; Navea et al., 2009a; Navea et al., 2009b). The presence of these compounds in aerosols could result in human exposure to species that are unlike the pure cyclic siloxanes previously investigated. Increased toxicity of soot and black carbon containing aerosols has been observed after oxidation in the atmosphere (Holder et al., 2012; Li et al., 2013). In these prior studies, aerosol particles induced multiple-fold increases in toxicity to cells once oxidized compared to preoxidation. Recent advances in detection identifying a high prevalence of siloxane-containing aerosols coupled with greater toxicity associated with the atmospheric oxidation of aerosols necessitate an understanding of the effects of aerosols containing photooxidized D5 on human health.

In this study, we generated aerosols composed of “daughter” compounds produced by photooxidation of D5 and assessed their impact on human lung cells. An oxidation flow reactor (OFR) was used to photooxidize cVMS under controlled conditions that mimic reactions in the atmosphere (Lambe et al., 2011). The physical characteristics of the aerosol from D5 oxidation were reported in Janecek et al. (2019) and here we use the same system with an *in vitro* aerosol exposure system to assess potential health implications of photooxidized cVMS aerosols. The aerosol exposure system (Vitrocell 12/6) enabled the passive deposition of aerosols onto the surface of lung cells cultured under air-interfaced conditions, mimicking aerosol exposures and culture conditions that occur in the lungs (Fiegel et al., 2003; Balharry et al., 2008; Wu et al., 2018). The ability of photooxidized cVMS aerosols to induce inflammation and toxicity in lung cells was evaluated. This work adds to a handful of studies that have characterized cytotoxicity of secondary aerosol using *in vitro* using air-liquid interface exposure systems (Arashiro et al., 2016; Lin et al., 2017; Yu et al., 2017; Chowdhury et al., 2018), and it is the only study to characterize cytotoxicity of secondary aerosol from volatile silicon precursors.

2. MATERIALS AND METHODS

Thirteen experiments were conducted (Table S1) using the apparatus shown in Figure 1. Experiments were about 4-h in duration, with additional time for system stabilization prior to aerosol characterization and/or cell exposure. Seven experiments included cell exposures

and six were performed for system characterization. The apparatus has three major sections described below – aerosol generation using a potential aerosol mass (PAM) type OFR, *in vitro* scell exposure in the Vitrocell system, and effluent characterization by SMPS, filter samples, and ozone monitoring.

2.1 Aerosol Generation and Characterization

Vapor-phase D5 was oxidized in a 13.3 L OFR using UV lamps to generate OH radicals following previously published methods (Kang et al., 2007; Kang et al., 2011; Lambe et al., 2011). A 5-LPM, HEPA-filtered, humidified air stream flowed past a capped piece of Teflon tubing containing liquid D5 (Sigma-Aldrich, 97% purity) maintained at 70°C in a water bath to vaporize the D5, and was fed into the OFR chamber through the feed port.

The OFR feed stream was maintained at 30% RH, as measured directly prior to the OFR feed port, by adjusting the fraction of dry air bypassing the humidifier. The OFR was operated in the OFR185 mode where both O₃ and OH are generated *in situ* from oxygen photolysis followed by reaction with water vapor. Flow from the sample outlet on the terminal side of the OFR left at a rate of 2.4 – 2.7 LPM, and the remainder of the outlet flow with more chamber wall exposure (ring flow, ~50%) was vented through the ring flow outlet, filtered, and exhausted through a lab hood. Additional details of the OFR-based aerosol generation system used in this work are reported in Janecek et al. (2019). OFRs generate substantial quantities of ozone (O₃), which was observed in preliminary experiments prior to the installation of annular denuders containing Carulite 200 (manganese dioxide/copper oxide catalyst; Carus Corp.). These experiments are listed in Table S1 but were not included in analysis. An activated carbon (Fisher Scientific; 6×14 mesh size) denuder was placed in-line to absorb organic gases from the OFR effluent. The activated carbon denuder dimensions were 25 cm outside diameter (OD), 20 cm inside diameter (ID) by 125 cm, while the Carulite denuders had dimensions of 14 cm OD, 1 cm ID by 70 cm, and 8 cm OD, 1.5 cm ID by 54 cm. The denuders were packed with material between the OD and ID. After the denuders, CO₂ was added to the sample stream at a level of 5% v/v of the total stream, which provides enough partial pressure to stabilize the sodium bicarbonate buffer in cell media to maintain physiological pH for the lung cells. The CO₂ level was measured at the end of the system using a CO₂ monitor (TSI 9555X with 982 probe). The CO₂-enriched sample stream then entered the Vitrocell glass manifold. Two sample outlets pulled a fraction of the flow off the main stream for cell exposures (~15 mL/min each) and the remaining fraction of the main stream exited the manifold for characterization. After characterization, the remaining flow was vented through a HEPA filter and passed through a Thermo 49i O₃ monitor before venting into a lab hood.

Particle size distribution and concentration in the manifold effluent were determined using a TSI 3936L85 scanning mobility particle sizer (SMPS; TSI 3785 condensation particle counter, TSI 3080 classifier, and 3077 Kr-85 2mCi neutralizer). The size distribution was sampled from 9.7 – 422 nm and scans were repeated every 135 s. Aerosol size distributions were converted to aerosol mass concentrations assuming spherical particles of the liquid D5 density (0.959 g/cm³) and spherical diameters equal to the reported electrical mobility diameters. For both SMPS and O₃ measurements, the data was processed by removing time

periods when the lines were opened for flow measurement or during supplemental sampling. SMPS data was additionally corrected by removing four scans that were flagged by the SMPS software as having too high of concentration or a CPC parameter out of range. The removed SMPS data constituted 63 min of data out of a total of 52 h of monitoring.

Particles were collected for evaluation using two sampling methods for electron microscopy. Samples downstream from the Vitrocell manifold were collected onto a carbon film supported by a 200 mesh nickel transmission electron microscopy (TEM) grid using a Thermophoretic Personal Sampler (TPS; model TPS100, RJ Lee Group, Inc.) (Leith et al., 2014). TPS samples were collected at 0.005 LPM for 25 min using hot and cold surface temperatures of 110°C and 25°C, respectively, and with 0.125 L total volume. TPS samples were analyzed at RJ Lee Group using a field emission scanning electron microscope (FE-SEM) with scanning transmission electron microscopy (STEM) capabilities (S-5500, Hitachi High Technology Corporation). Compositional information was obtained using an energy dispersive X-ray spectroscopy (EDS) system (Quantax 800, Bruker AXS Microanalysis GmbH) incorporating a silicon drift detector (Bruker XFlash 4030). Information about the size, morphology, concentration, and elemental composition of the collected particles was obtained as part of the analysis.

A second, passive collection of aerosol particles for microscopy analysis occurred inside the Vitrocell system. To characterize the distribution, morphology, and composition of particles depositing in the wells of the Vitrocell system, TEM grids were placed in the exposure chambers as has been reported previously with similar air-liquid interface exposures systems (Chortarea et al., 2015; Loret et al., 2016). Grids were prepared by coating 300 mesh Cu grids with formvar and carbon type-B, placed in clean transwells (cell culture inserts), and mounted in the exposure chambers. To maintain similar environmental conditions to cell exposures, culture media was maintained on the basolateral side of the inserts during collection. However, to reduce direct exposure of the grids to the media, the volume was reduced to 12 mL per chamber. Grids were exposed to aerosols for 4 h under the same conditions as the cell exposures. Due to the ubiquity of siloxane sources among people who use personal care products, it was possible that silicon-containing compounds could be exposed in the preparation and processing of formvar-coated grids. To verify that silicon-based materials collected on TEM grids were derived from the OFR system, a single grid in a clean transwell was placed next to the exposure system to sample lab air during particle collection in the Vitrocell system. After exposure, the grids were placed in a HEPA-filtered biosafety cabinet overnight to evaporate excess moisture. The samples were then analyzed for particle size and elemental composition using a JEOL JEM-2100F field emission TEM with an EDS system (Noran Nanotrace with NORVAR window). TEM images were analyzed using ImageJ software (Schneider et al., 2012) to determine the diameters of individual particles and EDS spectra were analyzed using Noran System Six.

Testing the time-resolved concentration of 0.3 – 20 µm particles with a GRIMM 1.108 aerosol spectrometer showed that agitation of the Carulite denuders could produce airborne dust in the system. Therefore, contamination from Cu and Mn containing dusts was tested for by chemical analysis of a filter sample, and by inspection of EDS spectra for Cu and Mn contamination. A 0.8 micron mixed cellulose ester (MCE) membrane filter was inserted

after the denuders and sampled for 90 h at a flow rate of 1.95 LPM. The filter was digested and analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) at the State Hygienic Laboratory of the University of Iowa using a modified NIOSH 7300 method. Briefly, filters were treated with 3 mL of concentrated nitric acid and heated at $98 \pm 5^\circ\text{C}$ in a hot block until the volume was reduced to approximately 0.5 mL. Another 3 mL of concentrated nitric acid was added and the volume was again reduced to 0.5 mL. Samples were removed from the hot block and the volume was brought up to 25 mL using 5% HNO_3 , 5% HCl . Analysis was performed on an ICP-OES instrument (Perkin-Elmer, Optima 5300DV) using external calibration with matrix-matched standards. Spectral interferences were minimized using inter-element correction factors and background correction.

To minimize non-D5 sources of elemental silicon causing false readings, silicon conductive tubing was minimized, with its only use in flexible adaptors to and from the Vitrocell manifold and connections to and within the SMPS system. A copper tube was used to connect the Vitrocell manifold effluent to the SMPS. The remainder of the system used Teflon tubing.

To measure the role D5 plays in particle formation in the system, experiments were performed with the OFR operating, but with a feed stream containing no D5. This was accomplished by closing the valve connecting the liquid D5 evaporation tube to the humidified air stream. The remaining experimental parameters were left unchanged, and particle analysis was performed using the SMPS.

2.2 Cell Culture Preparation

A549 cells (ATCC) from passages 5–15 were cultured in RPMI 1640 (Gibco) medium supplemented with 10% FBS (Atlanta Biologicals, lot C0089) and 1% penicillin-streptomycin, then incubated at 37°C in a humidified 5% CO_2 environment. Upon reaching confluence (5–7 days), cells were rinsed with phosphate-buffered saline (PBS) and treated with a 0.25% Trypsin-EDTA solution (Gibco) for 8 min to dissociate cells. Cells were suspended in culture medium and 0.5 mL aliquots were seeded onto 12 mm transwell inserts (Corning, 0.4 μm pore size, polycarbonate) at a concentration of 1.25×10^5 cells/mL with 0.53 mL of modified RPMI 1640 under the basal side of each transwell. The cells were grown to confluency under submerged conditions for 24 h. The apical media was then aspirated, and cells were allowed to adapt to an air interface for 12 h according to previously established methods (Kim et al., 2013; Jing et al., 2015).

2.3 Cell Exposures

The aerosol generation system was brought online 14–24 h prior to cell exposures (D5 temperature maintained at $40 - 55^\circ\text{C}$ using a water bath). Three hours before cell exposure, the D5 temperature was increased to its operational temperature of 70°C , and approximately 2 h before exposure, denuders and ring flow were added.

Six transwells (cell culture inserts) of 12 mm diameter were mounted in a Vitrocell 12/6 air-liquid interface exposure chamber (Vitrocell Systems, Waldkirch Germany) with 16 mL of culture medium under the basal side in each compartment. The chambers were maintained at 37°C by circulating heated water through the stainless steel jacket surrounding the

chambers. The exposure system was set up using the glass manifold distributor as described previously (Kim et al., 2013). The humidified, CO₂-enriched inlet stream passed into the Vitrocell manifold distributor at a flow rate of 2.4 – 2.7 L/min. The concentration of particles in the gas phase averaged 150 µg/m³ as measured by SMPS. The aerosol flow delivered to the cells (5 mL/min) was regulated by separate mass flow controllers (GFC17, Aalborg Instruments) downstream of the exposure chambers. Average flow rates through each of the Vitrocell chambers are provided in Table S2. The 4-h exposure time was selected from pilot studies exposing cells in the Vitrocell system to filtered air supplemented with 5% CO₂ at the same flow rates as used in the aerosol and OFR exposure studies. Exposure times greater than 4 h resulted in viability losses in these pilot studies, whereas no viability losses were observed at 4 h. The outlet streams from the six mass flow controllers were combined, filtered through a HEPA filter, and vented to a fume hood. Control experiments were run to determine the role of gas-phase components generated in the OFR by filtering out aerosols via a HEPA filter (TSI 1602051) placed after the denuders and exposing the cells to particle-free OFR effluent.

After controlled exposures in the Vitrocell or direct exposure to cells, the cells were gently rinsed with 500 µL of PBS and the PBS collected and centrifuged at 14,000×g for 30 min to remove cell debris. The supernatant was stored at –80°C for analysis of inflammatory markers. The cells were immediately processed to determine cell viability.

2.4 Cell Viability and Biomarker Assays

Cell viability was quantified using an MTS proliferation assay (Promega), which is a colorimetric assay for assessing cell metabolic activity. Prior to running the assay, transwells were treated with 250 µL of PBS-based, enzyme-free, cell dissociation buffer and incubated for 8 min at 37°C. Culture media (250 µL) was then added to each transwell, pipetted multiple times to loosen cells, and 100 µL aliquots transferred to a 96-well plate. Incubator controls were cells maintained in the incubator and positive controls were cells exposed to 250 µL of a 2% sodium dodecyl sulfate (SDS) solution in PBS. Each well was measured via absorption spectrometry at a wavelength of 490 nm (SpectraMax 384 Plus, Molecular Devices, and Epoch, BioTek). Absorbance values were normalized to controls to assess the relative viability of exposed to unexposed cells.

Cellular generation of inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), were measured using ELISA kits (Life Technologies). Positive controls were cells exposed to lipopolysaccharide (LPS) from Gram-negative bacteria, a known inducer of inflammation (Loret et al., 2016). A549 samples grown on transwells were exposed to 500 µL of 10–40 µg/mL LPS (L 2880, Sigma) in 90% PBS with 10% Hanks buffered salt solution (HBSS) for 6 h.

3. RESULTS AND DISCUSSION

3.1 Exposure Characterization

Oxidation intensity in OFR studies is typically expressed in terms of OH exposure (i.e., product of OH concentration and exposure time, molec s cm^{–3}) (Kang et al., 2011). The OH

exposure for this work was estimated at about 2.3×10^{12} molec s/cm³ while the OH concentration was approximately 1.4×10^{10} molec/cm³ as measured from SO₂ oxidation kinetics for chamber conditions of maximum light intensity and 30% RH. This OH exposure is equivalent to approximately 17.6 days of atmospheric aging assuming an average atmospheric OH concentration of 1.5×10^6 molec/cm³. This OH exposure oxidizes most of the feed D5, as it is slightly longer than the D5 OH atmospheric lifetime (~10 days) (Atkinson, 1991). OH exposure and oxidation conditions in the OFR are more extensively discussed in Janecek et al. (2019).

The OFR and denuder system produced a stable stream of aerosol particles and low ozone concentrations. Typical aerosol number and mass concentrations measured downstream of the denuders and the Vitrocell manifold by SMPS were 2.8×10^5 – 1.2×10^6 cm⁻³ and 82–220 µg/m³, respectively. This measurement reflects the concentration and size distribution of particles flowing through the Vitrocell after any losses caused by the denuders. Figure 2a shows typical temporal variability of mass concentration from the OFR over the course of a 4-h cell exposure period. The OFR aerosol output was stable during cell viability or microscopy experiments. Additional cell viability studies were done with the OFR operating and D5 fed into the OFR but with aerosol removal by an inline HEPA filter (Figure 2b). Additional tests were conducted to verify that both OH and D5 feed were required for aerosol production. Without D5, but with humid air feed and OFR UV lamps on, negligible particle counts were observed. Conversely, with D5 feed but with the UV lamps off, negligible particles were recorded.

The average available aerosol mass for deposition was 194 ng for each Vitrocell chamber, based on the average mass concentration of 161 µg/m³ and 4 hour experiment duration. Assuming a deposition efficiency of 50%, based on previous characterization of the Vitrocell system (Jing et al., 2015), the deposited aerosol is estimated at 86 ng/cm² of transwell or 97 ng per transwell. Uniform deposit (such that particles were neither concentrated nor depleted in the center where the 12 mm transwell was located) is based on characterization in Kim et al. (2013). Use of similar deposition efficiency as Jing et al. (2015) may be an upper limit on deposition, given the larger median diameter of the particles in this study, as well as sizedependent results from computational fluid dynamic and experimental studies of less than 10% deposition for submicron particles (Comouth et al., 2013; Lucci et al., 2018).

Figure 3 shows a comparison of electron micrographs of aerosols exhausted from the Vitrocell manifold via collection in the TPS sampler (a and b) and aerosols deposited in the Vitrocell system via collection by sampling onto TEM grids in the Vitrocell wells (c and d). Particle agglomerates or clusters of particles consisting of 2–30 primary particles, as well as a few lone particles, were observed at both sampling locations. In many of the images agglomerates can be seen where the primary particles have distinct borders (Figure 3c), while other agglomerates consisted of particles with poorly defined boundaries as illustrated in Figure 3d. Indistinct boundaries as shown in Figure 3d may indicate liquid or partially liquid particles. Volatility of the silicon-containing secondary organic aerosol was measured by Janecek et al. (2019) and found to be negligible.

Figure 4 shows the aerosol size distribution measured during experiment 12, where the particle size distribution and morphology was assessed by SMPS, TPS sampling, and passive TEM sampling. All size distributions are shown, after converting to a normalized concentration size distribution function ($dN/d\log D_p$). Two SMPS size distributions are shown—one uses the measured electrical mobility diameter (labeled SMPS), while the other uses a calculated equivalent volume diameter assuming agglomerates with primary particle diameter of 25 nm (based on the most prevalent primary particle size observed in the passive TEM analysis, see SI for more information). The aerosol size distribution mode in these analyses varied between 32–89 nm. The SMPS size distribution geometric standard deviation (GSD) was 1.64. For non-agglomerated spherical particles, the electrical mobility and volume equivalent diameter are equal. Accordingly, the shift in the size distribution based on equivalent volume diameter to smaller sizes shows the sensitivity of the distribution to particle morphology. Previous reports of Si-containing aerosols identified particles in the 20–50 nm range (Phares et al., 2003; Bzdek et al., 2014), which are similar to the particles produced in the OFR with D5.

Projected area diameters were measured using ImageJ on the TPS samples for 1412 structures including agglomerates. The SMPS and TPS size distributions are relatively similar in total number and size distribution. The diameters obtained from the passive TEM samples ranged widely from about 10–100 nm, with a count median diameter of the 214 particle population of 31 nm. As the TEM sizes are based on measurement of primary particles making up the agglomerates using ImageJ (projected area diameter of 214 particles), agreement with the distributions based on the agglomerates is not expected.

The chemical composition of particles containing siloxanes was confirmed in both the TPS samples and passive TEM samples measured by EDS, both indicating strong silicon and oxygen signatures (Figure 5). Silicon and oxygen were uniformly distributed within the particles at high concentration compared to the background, whereas carbon, a component of the grid, was uniformly distributed across the scan area (Figure 5b). This composition is consistent with aerosols formed primarily from oxidized derivatives of D5. Background peaks consistent with the nickel TPS grid and the copper passive TEM grid were evident in the spectra. In addition, the carbon film on the TPS grids and formvar coating on the passive TEM grids created background carbon signals.

O₃ was removed from the reactor outlet stream using Carulite 200 denuders and an activated carbon denuders as ozone is known to damage lung cells (Alink et al., 1980; Uhlson et al., 2002; Poma et al., 2017). Typical O₃ concentrations downstream of the denuders was 2.5 ppb (Figure S1). Peak O₃ concentrations (20-s averaging) were 5 ppb when limiting analysis to cell exposure periods only, and 43 ppb considering all denuded OFR effluent. As the Carulite used in the denuders is a manganese dioxide/copper oxide catalyst, we further tested the OFR effluent to ensure no catalyst contamination was present. ICP-OES analysis of filter samples found both components to be below detection limits, 0.3 and 0.5 $\mu\text{g}/\text{m}^3$ for Cu and Mn, respectively.

EDS analysis of deposited aerosols (Figure 5) also indicated no signs of contamination by Cu- and Mn-containing particles. No Mn was detected in any of the six TPS samples. A

small Cu peak was observed for some samples, likely originating from the sample holder. TEM-EDS analysis of the passive TEM samples collected in the Vitrocell chamber also did not detect Mn. A strong Cu signal was present in the spectra due to generation of extraneous X-rays from the Cu TEM grid.

3.2 Cell Responses to Oxidized D5 Aerosols and Gaseous Products

Exposure to OFR effluent reduced viability in A549 cells compared to no-exposure controls (Figure 6). Viability decreases relative to no-exposure controls were seen with secondary aerosols generated from D5, and from OFR effluent where the aerosol was filtered out; the difference between these cases was not statistically significant. Furthermore, dose-dependence in cell toxicity was not observed (Figure 6b) in three experiments with doses ranging from 54 to 116 ng/cm². Losses in viability relative to incubator controls were observed when the OFR product stream was filtered to remove aerosols. When cells were exposed to OFR effluent, but with the OFR lamps not on, no reduced viability was observed (data not shown).

Collectively, these results imply that OFR chemistry produces one or more gaseous products that are both toxic to cells and not fully removed by the denuders. Important products of the OFR include O₃ as well as hydroxyl (OH) and peroxy (HO₂ and RO₂) radicals (Kang et al., 2007; Lambe et al., 2011). While measurement of O₃ downstream of the denuder was performed, other gas phase species concentrations such as peroxy radicals were not measured downstream of the activated carbon and Carulite denuders. These reactive oxygen species are known to damage cells (Rojanasakul et al., 1993; Spiteller, 2006). Thus, cell exposure to OH and HO₂ is a likely hypothesis for the reduced viability in the absence of particles. The results presented here suggest that aerosols produced from oxidized D5 derivatives are not likely to be highly toxic, though they may be responsible for some of the observed decrease in cell viability.

Comparing to similar studies in the literature, we have identified several studies using secondary organic aerosol (SOA) followed by *in vitro* cytotoxicity using an air-liquid interface delivery systems. Of these studies, most use environmental chambers (a.k.a. smog chambers) for aerosol generation (Arashiro et al., 2016; Lin et al., 2017; Yu et al., 2017), while one study used the shorter residence time (and higher oxidant levels) of an oxidative flow reactor (Chowdhury et al., 2018) similar to in this study. The studies using smog chambers for SOA generation in combination with air-liquid interface delivery systems reported deposited doses of 0.06 to 0.18 µg/cm², comparable to the 0.054–0.116 µg/cm² reported herein. Tests with primary aerosols such as tobacco smoke, metal fumes, carbon black, and dusts, are often performed at much higher doses; see for example Rach et al. (2014), Adamson et al. (2014), and Kim et al. (2013), which report values across a range spanning from 1 to 100 µg/cm².

In Chowdhury et al. (2018), an OFR is used for oxidation of the SOA precursors alpha pinene and naphthalene, followed by *in vitro* cytotoxicity studies with a CULTEX RFS system. In contrast to the current work, Chowdhury et al. reported a particle-specific response, with no observed effects on cells when OFR effluent was filtered. Differences that could be explored to understand different outcomes include the SOA precursor, dose, and

aerosol generation system details (specifics of driers, denuders, size distribution, etc.). For example, the reported doses on a per area basis in Chowdhury et al. were an order of magnitude or higher than those reported herein. Additional differences that may be warrant future investigation include different air-liquid interface exposure system (CULTEX RFS vs. Vitrocell), use of the OFR185 vs. OFR254 mode for photooxidation, presence or absence of potential fields for electrodeposition, and a different approach to viability measurements (testing 24 h after exposure rather than immediately). Delayed cell assays allow for longer interaction times with deposited aerosols in the absence of OFR gas effluents.

Figure 6 indicates a large range of variability in experiments with the OFR lights on. Each marker on the figure represents one Vitrocell exposure chamber during one experiment. Experiments typically used five Vitrocell chambers, although some used four chambers. The experiment count (n) values in the Figure 6 caption (and used for statistical significance testing) refer to the number of experiments. The number of marker points in Figure 6a is approximately 5n due to the five Vitrocell chambers. Each marker is calculated by averaging viability across multiple replicates in the well-plate used for the assay (5 replicates were typically used). The variability in the points shown in Figure 6 reflect most, but not all sources of variability, discussed below, in our experiments. However, we remain conservative in our statistical significance testing by using a conservative selection for n, without increasing n to reflect replicate Vitrocell channels or replicant well-plates in the assay.

Sources of variability in our experiments are (a) between experiments, (b) between Vitrocell chambers within an experiment; (c) between replicates sharing the same experiment and Vitrocell chamber; (d) variability in the absorbance characterizing viability of unexposed cells during an experiment; (e) variability in blank absorbances (due to the wellplate, buffer and cells). Of these five sources of variability, (a) and (b) were significant and are shown by the scatter in Figure 6. Variability due to differences in replicate live control absorbance (variability type d) was significant, but is not reflected in in Figure 6 (markers are based on average live control absorbance). Variability due to differences between replicate well plates (type c) was smaller than the previously mentioned sources of variability and is also not reflected in Figure 6 (markers are based on the average of ~5 replicates). Variability due to blanks (type e) was negligible.

Cellular stress and damage may occur in cases where death does not occur; in those instances, cells may exhibit signs of inflammation indicative of that stress (Gualtieri et al., 2010). Two markers of inflammation that are known to be released as a stress response in A549 cells are TNF- α and IL-6 (Crestani et al., 1994; Yang et al., 2002). TNF- α secretion increases flow from the blood to the tissue to bring innate immune proteins and immune cells into the space, while IL-6 activates white blood cells and signals antibody production (Murphy et al., 2012). After 4-h exposure to oxidized-D5 aerosols, no increase in secretion of TNF- α or IL-6 was observed. However, after exposure to LPS, a dose-dependent response of IL-6 was elicited (Figure 7). LPS failed to elicit a measurable response of TNF- α in the same cases (data not shown). These results indicate that the cytotoxicity measured in acutely exposed cells was not accompanied by a pro-inflammatory response.

For future studies with OFR generation of secondary aerosols for toxicological testing, we recommend expanded characterization of radicals, oxidants and organic gases in the gas-particle mixture being used in the cell or animal exposure. Use of an in-line GC-MS, or additional testing of oxidative potential of gas and aerosol species, may elucidate the cause of the observed toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Oxidative flow reactor used to study effects of secondary aerosols on lung cells
- Nanoparticulate aerosols generated from OH oxidation of D5, a cyclic siloxane
- Acute exposures to 54–116 ng/cm² achieved using an air-liquid interface system
- Cytotoxic and proinflammatory effects marginal or absent at these doses

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Figure 2.

Characterization of aerosol particles generated from the OFR. (a) Representative plot of mass concentration throughout a single experiment (from SMPS). (b) Average concentrations (from SMPS) of aerosol particles formed under different reactor conditions (error bars are equal to the standard deviation of the individual concentrations measured every 135 s during each experiment).

Figure 3. SEM and TEM images establishing particle morphology of the generated particles. SEM images of TPS samples are shown for (a) bright field and (b) secondary electron image, while TEM images of passive TEM samples are shown in (c) and (d). Panels (c) and (d) contrast two types of particles imaged by TEM—some had distinct boundaries, while others had indistinct borders.

Figure 4.

Intercomparison of particle size distributions and size-resolved concentrations from experiment 12, which included SMPS, TPS sampling, and passive TEM sampling. The SMPS diameters are electrical mobility diameters; the SMPS (agglomerates) diameters are equivalent volume diameters of the agglomerate converted to number concentration with an assumed primary particle size of 25 nm; the TPS diameters are projected area diameters of agglomerates; the passive TEM diameters are projected area diameter of primary particles (not agglomerates). The passive TEM size distribution has been normalized to a maximum of $6 \times 10^5 \text{ cm}^{-3}$.

Figure 5.

EDS analysis of the chemical composition of particles from (a) TPS sample of Vitrocell exhaust and (b) passive TEM sample of aerosols deposited in Vitrocell wells. (a) EDS analysis of a particle agglomerate (see inset) collected during TPS sampling is shown in red and background grid is shown in blue. (b) Original electron micrograph illustrating the EDS sample region selection (blue crosshair) and comparative background region (orange crosshair) (bar = 50 μm); element mapping of silicon, oxygen, and carbon (bar = 50 μm);

and EDS spectra of a particle obtained by passive TEM sampling confirmed that deposited aerosols are derived from siloxanes.

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Figure 6.

(a) Relative viability of cells exposed to filtered gases from the OFR (n=4), or OFR-generated aerosols (n=3) compared to no exposure and SDS controls (***) $p < 0.001$). (b) Effect of dose dependent aerosol-exposure on relative viability of cells.

Figure 7.
Concentration of IL-6, a cytokine marker for inflammation, in supernatants collected from A549 cells after exposure to aerosols or gases generated in the OFR reactor.