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Effect of collection methods on combustion particle physicochemical properties and their biological response in a human macrophage-like cell line

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Abstract

In vitro studies are a first step toward understanding the biological effects of combustion-derived particulate matter (cdPM). A vast majority of studies expose cells to cdPM suspensions, which requires a method to collect cdPM and suspend it in an aqueous media. The consequences of different particle collection methods on particle physicochemical properties and resulting biological responses are not fully understood. This study investigated the effect of two common approaches (collection on a filter and a cold plate) and one relatively new (direct bubbling in DI water) approach to particle collection. The three approaches yielded cdPM with differences in particle size distribution, surface area, composition, and oxidative potential. The directly bubbled sample retained the smallest sized particles and the bimodal distribution observed in the gas-phase. The bubbled sample contained ~50% of its mass as dissolved species and lower molecular weight compounds, not found in the other two samples. These differences in the cdPM properties affected the biological responses in THP-1 cells. The bubbled sample showed greater oxidative potential and cellular reactive oxygen species. The scraped sample induced the greatest TNF α secretion. These findings have implications for in vitro studies of air pollution and for efforts to better understand the underlying mechanisms.

Keywords

Collection methods; Combustion particles; Physicochemical properties; Particle size distribution; Jet fuel surrogate; Reactive oxygen species; Inflammatory response; Nanotoxicology

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Introduction

Elevated levels of fine particulate matter (PM_{2.5}) are strongly associated with adverse human health effects, including heart attacks, strokes, decreased lung function, and premature death. [1,2] PM_{2.5} is a complex mixture of combustion-derived particulate matter (cdPM), dust, salt and secondary aerosols, which are formed through atmospheric reactions. cdPM can be responsible for over half of ambient PM_{2.5}, particularly in urban areas. [3,4] An important approach to understanding the mechanisms by which PM causes adverse health effects involves in vitro studies of cellular responses to cdPM.

A number of interesting in vitro studies have demonstrated that particle size, composition (especially content of polycyclic aromatic hydrocarbons, PAHs), and surface area have major impact on cellular responses. For example, some studies have found an increased inflammatory response associated with finer fractions of ambient PM. Li et al. [5] compared the effect of PM_{0.1} with that of PM_{0.1–2.5} and PM_{2.5–10} in in vitro assays. They found that PM_{0.1} induced greater oxidative stress in both macrophage (RAW 254.7) and epithelial (BEAS-2B) cell lines compared to the coarser particles. Similarly, using ambient samples collected from sites influenced by traffic, Steenhoff et al. [6] found that PM_{2.5} and PM_{0.18} induced greater pro-inflammatory activity compared to PM_{2.5–10} in a murine macrophage cell line. Schwarze et al. [7] found that for particles with a similar or identical composition, particle surface area was a more important factor in predicting biological responses than particle mass. Consequently, smaller particles induce greater inflammatory responses than larger particles on mass basis because of their higher surface to mass ratio. In addition, Schwarze et al. [7] found that PM composition is a key factor in PM's inflammatory activity. cdPM contains adsorbed species, such as unburned fuel and PAHs, and some studies have associated increased inflammatory responses and oxidative stress with greater PAHs content, [8,9] although other studies suggest that the relationship between inflammatory response and PAHs content is more complex. [10]

In the vast majority of such in vitro studies, cells are exposed to cdPM as a suspension. This approach involves three main steps: particle collection, transfer, and subsequent suspension in aqueous media. Combustion particles can be collected in a variety of different ways, including by filter, [8,11–18] impactor, [19–21] scraping from an engine tailpipe, [22,23] electrostatic precipitation, [9,24] and direct bubbling in DI water, ethanol, or other liquid. [25–29] However, very little is known about the consequences of the different particle-collection approaches on the PM's physiochemical properties or the biological effects elicited in response to the PM, although some data suggest that particle collection and application methods can alter cdPM toxicological properties. [21]

This study sought to determine whether particle collection method would affect the physicochemical properties of cdPM and how these differences, in turn, affected biological responses. Specifically, using a single fuel in a flat-flame burner under constant combustion conditions, we compared three different collection methods: 1) collection on a filter, 2) scraping from a cold impactor plate, and 3) direct bubbling of gas-phase combustion products into DI water. We determined the effect of these collection methods on particle size

distribution in the suspended phase, chemical composition, particle shape and structure, oxidative potential, potential to cause intracellular reactive oxygen species generation, pro-inflammatory potential (TNF α secretion) and acute cytotoxicity using human blood monocytes (THP-1) that were differentiated into macrophage-like cells, as a model for airway macrophage interactions with cdPM.

Materials and methods

Samples collection and preparation

Particles were generated from a premixed flat-flame burner combusting a jet-fuel surrogate at an equivalence ratio of 2.5 (fuel flow rate: 60 mL/hr; air: 5.4 L/min). The jet-fuel surrogate (Hex-12 in Table 1^[30]) and the consistent combustion conditions permitted the generation of particles with reproducible physical and chemical properties. A custom-made vaporizer converted the liquid fuel to the gas phase, with a consistent chemical composition before and after the vaporizer, as confirmed by gas chromatography-mass spectrometry (GC-MS) of the condensed fuel after the vaporizer (Table 1S, supporting material). A nitrogen shroud and quartz housing minimized atmospheric effects on the flame in a setup similar to that used by Ghazi et al.^[31] The particles were collected using an open-end probe at a height of 15 cm above the burner (post-flame region) as shown in Figure 1. The three collection methods were:

1. **Bubbled (Bub):** A vacuum pump drew particles through an impinger filled with 50 mL of HyClone™, endotoxin-free, cell-culture grade water (Fisher Scientific, Waltham, MA). The impinger was placed inside an ice bath and was sonicated continuously while collecting sample. The sample was collected for 1 hour.
2. **Filter-collected (Fil):** A vacuum pump pulled gas-phase particles through a 200-nm polytetrafluoroethylene (PTFE) filter (Whatman Ltd). The filter was sonicated for 2 min to suspend particles from the filter into 50 mL of endotoxin-free, cell-culture water, after which the filter was removed. Subsequently, the suspension was sonicated for 30 min.
3. **Scraped (Scr):** A plate cooled by a water jacket on one side was placed at the height of 15 cm above the burner surface, causing particles to deposit on the plate. The temperature at 15 cm above the burner was 550–600°C, a representative temperature of an engine tailpipe. The plate was scraped to collect particles, which were suspended in 50 mL endotoxin-free, cell-culture water. The suspension was sonicated for 30 min.

After sonication, each of the samples was pipetted into a 50 mL sterile vial for further analysis or cellular studies. The pipetting occurred in the middle of the sample and excluded any particles that were either floating on the DI water surface or had precipitated to the bottom of the vial. The mass concentrations of the samples were measured by quartz crystal microbalance (QCM, discussed in the next section), and a 15 mL stock solution of 220 μ g/mL was prepared for each sample. We examined the composition and the oxidative potential of the samples and of the soluble portion of the samples, which were obtained by

passing the samples through a filter (200-nm pore size syringe filter for GC-MS and 20-nm pore size syringe filter for the dithiothreitol, DTT, assay). The 20-nm filtered samples were called: FBub for the bubbled sample, FFil for the filter collected sample, and FScr for the scraped sample.

Analytical methods

Mass concentration measurement—Mass concentration of all three samples was estimated using a quartz crystal microbalance (QCM200, Stanford Research Systems).^[32] A 0.5 µL drop of a sample was carefully placed at the center of the crystal with the help of a micro-syringe and dried inside an incubator (Model 10E, Quincy Lab, Inc.) at a constant temperature of 25°C. After complete evaporation of the drop and a minute after the frequency signal stabilized, the change in resonance frequency of the crystal was measured. Mass concentration was estimated from the Sauerbrey equation:^[33]

$$\Delta f = -C_f \cdot \frac{\Delta m}{A}$$

(1)

where f was the observed change in the resonance frequency (Hz), m was the change in the mass (µg), C_f was the sensitivity factor of the crystal (56.6 Hz µg⁻¹ cm² for a 5 MHz AT-cut quartz crystal at room temperature), and A was the piezoelectrically active area (the area between the electrodes; cm²). The area A of the crystal used in this study (5MHz, AT-cut, plano plano, Cr/Au, diameter of 1 inch) was 0.28274 cm² (radius of 0.3 cm). Although the deposition of the particles on the surface of the crystal was somewhat non-uniform, the effect of Gaussian radial sensitivity on mass was ignored since the drop was within 1 mm of radius from the center of the crystal.^[32] All the samples were run in triplicate. The micro syringe was rinsed with ethanol between samples. The percent of dissolved species in each of the samples was estimated from the QCM mass measurements of FBub, FFil and FScr and the corresponding mass concentration of the original (Bub, Fil, and Scr) sample (Equation 2).

$$\text{Percent dissolved species} = \frac{\text{mass concentration 20 nm filtered sample} \left(\frac{\mu\text{g}}{\text{mL}} \right)}{\text{mass concentration original sample} \left(\frac{\mu\text{g}}{\text{mL}} \right)} \times 100$$

(2)

Dynamic light scattering (discussed in the next section) confirmed the absence of particles (Table 2S, supporting material) in the FBub, FFil, and FScr samples.

Gas-phase particle distribution—A scanning mobility particle sizer (SMPS, TSI, Inc., Shoreview, MN, model 3080) with a nano-differential mobility analyzer (TSI model 3085) and ultra-fine condensation particle counter (TSI model 3025 A) measured the particle size distribution (PSD) in the gas-phase. The particles were drawn through an open-end probe at the height of 15 cm above the burner (post-flame region) using an eductor (#15, Fox Venturi Mini Eductors; motive air pressure set at 20 psig), and the sample was further diluted with another eductor (#15, Fox Venturi Mini Eductors; motive air pressure set at 20 psig) to a final dilution of 100:1. The inlet aerosol flow rate and sheath rate of SMPS were set at 0.4 L/min and 1 L/min, respectively.

Suspended phase particle size distribution and surface area—The PSD in the final samples was determined using multi-angle light scattering (MALS, DAWN HELEOS II, Wyatt) connected in-line with the asymmetrical flow field-flow fractionation (AF4) flow channel (Section 1S, supporting material). The MALS signal was processed using ASTRA V 5.3.4 software to obtain the root mean square (rms) radius and number density distribution. The Debye model was used for estimating rms radius. The number density distribution was estimated using the Sphere model and a refractive index (RI) for soot of 1.65 (real RI part for diesel particles) at room temperature.^[34] The samples were characterized in batch dynamic light scattering (DLS, Möbius, Wyatt) and processed for mean hydrodynamic radius using Wyatt's DYNAMICS 7.8.0.26 software. The surface area per unit mass was calculated from the rms PSD, fractal dimension, and known primary particle diameter (Section 2S, supporting material).

Transmission electron microscopy—A 0.5 μ L drop of each sample (35 μ g/mL) was placed on a copper grid (200 mesh Cu, Lacey F/C, Ted Pella Inc.) and dried in the incubator at 30°C until the drop fully evaporated. Transmission electron microscopy (TEM) images were taken at various magnifications using JEOL 2800 (accelerating voltage of 200 kV).

GC-MS—The composition of samples was compared by hexane extraction of the materials, followed by GC-MS analysis (Thermo Scientific 1310 Gas Chromatograph equipped with a Thermo Scientific ISQ LT Single Quad Mass Spectrometer). The samples were prepared from 3 mL of the sample stock solution, which was diluted to 10 μ g/mL. Half of each diluted stock solution was filtered through a 200-nm syringe filter (Whatman Ltd.) to determine the composition of the soluble fraction of the sample. A 30 mL aliquot of each sample (10 μ g/mL) was placed in a 250 mL amber vial. A 10 μ L aliquot of 35% aqueous ammonia was added by pipette and the sample was then diluted with DI water to 50 mL. A total of 15 mL of hexane was added to the 50 mL solution and the vial was shaken for 20 hours using a rotary shaker. Subsequently, the upper 10 mL of the organic phase was pipetted into a 25 mL glass beaker and was concentrated to 250 μ L under a gentle flow of nitrogen. The concentrate was transferred to 2 mL septa closure vials for GC-MS analysis. The same procedure was followed for 200-nm filtered samples to characterize the dissolved chemical species in the solvent (DI water).

DTT assay

The cell-free oxidative potential of each sample was tested using the DTT oxidation assay, wherein DTT consumption is directly proportional to the oxidative potential of a sample. [35] The samples were prepared from 3 mL of the stock solution of each sample, which was diluted to 35 µg/mL. Half of each diluted stock solution was filtered through a 20-nm syringe filter (Anotop, Whatman Ltd). These 20-nm filtered samples were called: FBub for the bubbled sample, FFil for the filter collected sample, and FScr for the scraped sample. A total of 100 µL of each sample (35 µg/mL) was mixed with 25 µL of 0.5 mM DTT (freshly prepared in phosphate-buffered saline, PBS) and was incubated at 37°C for time intervals of 0, 30, 60 and 90 min. After each interval, the reaction was quenched by adding 25 µL of 0.75 mM of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) to the reaction mixture. The unreacted DTT reacted with DTNB to form 2-nitro-5-mercaptobenzoic acid, which was measured by absorption of visible light at 415 nm. The assay was performed in triplicate in a 96-well plate. The DTT consumption rate (pM/µg of sample/min) was measured from the slope of DTT consumption versus time. The effect of DI water (negative control) on the DTT consumption was accounted for in reporting the final DTT consumption rates. Hydrogen peroxide (ACS Reagent, Honeywell, USA) at a concentration of 35 µg/mL was used as a positive control.

In vitro studies

Cell culture—THP-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in complete growth medium (Roswell Park Memorial Institute medium 1640 with L-glutamine and 0.05 mM 2-mercaptoethanol (Bio-Rad, Inc. Hercules, CA) and 10% Fetal Bovine Serum (GE Healthcare Bio-Sciences, Inc. Marlborough, MA)). THP-1 cells are a pro-monocytic cell line that are differentiated to pulmonary macrophage-like cells in 200 nM of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, St. Louis, MO), as described by Daigneault et al. [36] and O'Reilly et al. [37] Cell cultures were incubated at 37°C in 5% CO₂ and 95% humidified air and kept in the logarithmic phase of growth throughout all experiments.

Cell treatments—Cells at 70–80% confluence were exposed to cdPM, collected using each of the three approaches, in fresh complete growth media. cdPM was either collected directly in DI water (described in the section on sample collection) or suspended in DI water and sonicated immediately before addition to the cells. The cdPM dose was 33 µg/mL in fresh complete growth media (12.4 µg /cm² for reactive oxygen species (ROS) and 8.25 µg /cm² for ELISA). An exposure time of 24 hr was used to capture short-term, acute responses induced by the cdPM samples. The control for all experiments was cells treated with media and a corresponding amount of DI water (15% by volume, which corresponds to the 33 µg/mL dose of cdPM), which was confirmed to be non-toxic by CCK-8 assay (described under cell viability).

ROS and inflammatory response—Reactive oxygen species and inflammatory response were evaluated with cellular assays of intracellular ROS and ELISA, respectively. Intracellular ROS was measured using the OxiSelect™ Intracellular ROS Assay Kit (Green

Fluorescence; Cell Biolabs, Inc. San Diego, CA, USA) according to the manufacturer's instructions. [38] The cells were seeded in 96-well black wall/clear bottom plates (Corning, Inc., Corning NY) and treated with the cdPM for 24 hr. Fluorescence intensity indicative of the formation of ROS was measured using excitation and emission wavelengths of 480 nm and 530 nm, respectively with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). ROS production is expressed as relative fluorescence intensity normalized to the fluorescence of the negative control (media with an equivalent amount of sample DI water).

Secretion of tumor necrosis factor (TNF α) protein in response to cdPM treatment was measured in cell supernatants by ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer protocols. All media samples were cleared of debris by centrifugation at 10,000 xg for 10 minutes, aliquoted and frozen. The measurements were carried out on media samples that had gone at most a single freeze-thaw cycle.

Determination of cell viability—Cell viability was determined using Cell Counting Kit-8 (CCK-8) assay kits (Dojindo Molecular Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. THP-1 cells were seeded at 20,000 cells per well in 96-well plates and differentiated in 200 nM PMA for 72 hr and allowed to rest for 48 hr prior to treatment. [36,37] Cells were exposed to fresh-prepared cdPM (33 μ g/mL) and incubated for 24 hr. The media containing particles were removed, and the cells were incubated in 100 μ L of a medium solution containing 10% (v/v) CCK-8 reagent. The cells were incubated at 37°C for a further 3 hr. The absorbance was obtained by scanning with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Each experiment was performed at least three times in triplicate. The cells treated with 10% DMSO served as a positive control group, and cells treated with the 15% (volume based) DI water in media served as the negative control. Cell viability was calculated according to the following formula: cell proliferation (%) = absorbance of the experimental group/absorbance of the negative control group $\times$ 100. The results were confirmed by visual inspection.

Quantification of apoptosis and necrosis by flow cytometry—The effect of cdPM treatment on cell viability, apoptosis and necrosis were determined using Annexin V-FITC and propidium iodide (PI) staining Kit (Invitrogen™, Eugene, OR) by flow cytometry. THP-1 cells were seeded into 12-well plates and treated with cdPM. After 24 hr of exposure, cells were washed with PBS, trypsanized to detach the cells, and re-suspended in Annexin V buffer according to manufacturer's recommendations. A total of 100 μ L of the cell suspension was mixed with 5 μ L of FITC Annexin V and 1 μ L of 100 μ g/mL PI.

The mixture was incubated in the dark for 15 min at room temperature after which an additional 200 μ L of Annexin V buffer was added. Samples were then placed on ice and analyzed by flow cytometry (FACSCanto BD Biosciences San Jose, CA) at emission wavelengths of 530 and 670 nm, respectively, within one hour. Assays were carried out in triplicate (33 μ g/mL dose for 24 hr) with 10,000 cell events for each analysis. Population means were aligned via the use of single-color controls and compensation by the analytical software (Flowjo Version 10).

Data analysis—Statistical analysis was performed using one-way analysis of variance (ANOVA) with astatsa.com online software and Tukey's honest significant difference (HSD) post-hoc test to compare differences between all sample means. Comparisons were made between control and treated groups as noted in figure legends.

Results and discussion

Table 2 summarizes the sample physicochemical properties and compares the relative biological outcomes of the different samples.

Effect of cdPM collection method on sample physicochemical properties

The three different cdPM collection methods resulted in significant differences in the physicochemical properties of the resulting samples. In the gas phase, the jet-fuel surrogate combustion particles exhibited a bimodal distribution with peaks at mobility radii of 47.5 nm and 108 nm (Fig. 2a). Particles from ethylene flame (collected at heights >1.5 cm above the burner) are commonly bimodal, [39,40] and gasoline- and diesel-engine-generated particles can also exhibit a bimodal distribution. [41,42] After collection and suspension, only the Bub sample retained this bimodal distribution, with peaks at rms radius of 41.1 nm and 60 nm (Fig. 2b). Also, Bub had the smallest mean rms radius as compared to Fil and Scr (Table 1). The same trend was observed for the hydrodynamic radius (Table 1). Agglomeration associated with filtering or plate collection is a likely explanation for the greater mean radius in the Fil and Scr samples. Our results showed that sample collection methods affected the suspended-phase PSDs. They also emphasize the importance of characterizing cdPM in the exposure media rather than the gas phase, although most studies only report gas-phase PSD. The techniques used for characterizing the particle size distributions in the gas phase (SMPS based on electrical mobility) differ from those used in the liquid phase (MALS based particle light scattering). Consequently, gas-phase particle sizes are presented in terms of electrical mobility radius, and suspended-phase particle sizes are presented in terms of rms.

Several studies suggest the importance of PM surface area in biological responses, with greater surface area being associated with greater pro-inflammatory potential. [7,43] Scr and Fil had higher surface areas per unit mass than Bub (Table 1). In this study, all treatments used a constant mass dose. However, the dissolved species comprised approximately 55% of the Bub mass, but less than 35% and 20% of the Fil and Scr samples, respectively. Furthermore, the dissolved species did not contribute to particle counts or surface area. Consequently, the Bub sample, although smaller in size, had lower particle numbers and less surface area to interact with cells compared to the other two samples.

All the primary particles in all samples exhibited an "onion-like" fringe arrangement (Fig. 3). This onion-like fringe arrangement has been previously reported in soot from diesel and jet engines. [44,45]

The collection methods also affected the chemical characteristics of the samples. As observed from the GC-MS results, the Bub samples showed the presence of lower molecular-weight species, such as phenol, benzaldehyde, and styrene (Fig. 4) that were not

found in the other two samples. All samples contained PAHs, but the Bub and Fil samples contained greater concentrations than the Scr sample. The lower concentration of adsorbed volatile compounds in Scr was likely caused by “cooking” of particles due to the collection temperature of 550–600°C, which is representative of the temperature at an engine’s tailpipe. When each sample was filtered with a 200-nm filter, the 200-nm filtered-Bub sample showed similar peaks compared to the original Bub sample. However, the 200-nm filtered-Fil and 200-nm filtered-Scr samples showed fewer peaks than Fil and Scr samples, respectively (Fig. 5). This suggests that the Bub sample contained a greater fraction of dissolved species (~50% of total mass, as found by the QCM of 20-nm filtered Bub sample), whereas the Fil and Scr samples contained more particle-bound species.

Effect of sample physicochemical properties on oxidative potential and ROS

The cell-free oxidative potential is directly proportional to the DTT consumption rate,^[35] which is related to a sample’s chemical composition. In this study, each of the three samples contained different fractions of dissolved organic species and different chemical characteristics, and these differences were associated with significant differences in oxidative potential. Figure 6 shows that the original Bub samples exhibited the greatest oxidative potential, with a statistically significant increase compared to Scr ($p<0.05$). The figure also shows that the dissolved fraction of the Bub sample contributed as much or more than the particle phase to the oxidative potential of this sample. In fact, we found no significant difference among the particle-phase contributions of the three samples, as estimated by the difference between the DTT consumption of the original sample and the corresponding filtered sample. These results suggest that the soluble organic species are a large contributor to oxidative potential, which agrees with previous studies of oxidative potential associated with combustion particles^[46] and ambient particulate matter.^[47]

Others have found that both DTT consumption by cdPM and ROS generation by cells exposed to cdPM are related to the presence of soluble organic material and PAHs.^[35] We also found that intracellular ROS results showed a trend similar to the cell-free oxidative potential, with the Bub sample inducing significantly ($p<0.01$) increased intracellular ROS formation in THP-1 cells compared to Fil and Scr (Fig. 7b). The Bub sample contained more soluble organic material than the other two samples, and these compounds may either directly generate ROS or generate ROS following metabolic biotransformation. All samples increased ROS relative to the DI water control. It should be noted that none of the samples caused significant toxicity, as measured by CCK-8 assay at the cdPM concentration used in this study (33 µg/mL, Fig. 4S, supplementary material). Our results agree with an earlier study that found diesel particulate matter and the corresponding organic extracts induced ROS production,^[48,49] but carbon black particles, which represent the non-soluble fraction of cdPM, did not induce ROS.^[48]

Apoptosis and Necrosis

In vitro exposure to diesel PM can induce early apoptosis and cell death.^[50] Our flow cytometry results provided additional insight into the effect of the cdPM on the different phases of the cell cycle. The two-dimensional flow cytometry plots (number of cells versus

the area under the peak) were divided into three quadrants of interest: late apoptotic cells/necrosis (Annexin⁺ and PI⁺), normal viable cells (Annexin⁻ and PI⁻) and early apoptotic cells (Annexin⁺ and PI⁻).^[51] The distribution of cells within these quadrants was compared between cells treated with cdPM and those in the negative control sample (media treated with the same dose of DI). The treatments (at a dose of 33 µg/mL) resulted in a maximum of 15% late apoptotic/necrotic cells. The Scr sample induced a significant increase in early apoptotic cells compared to the control ($p < 0.01$, Fig. 7c), while the other two samples did not show significant differences relative to the control. These results suggest that collection methods and the related physiochemical properties can influence macrophage cell apoptosis.

Inflammatory response

The secretion of pro-inflammatory cytokines by lung cells and macrophages in response to cdPM is widely reported in the literature.^[5,7,9,12,52,53] All three cdPM samples stimulated TNF α secretion (Fig. 7d). Accumulation of particles in macrophages activates these cells to secrete cytokines, such as TNF α . The Scr sample induced the greatest inflammatory response, and this sample had the greatest particle surface area (Table 1), which may promote more extensive interactions with the cell surface and cellular uptake of the particles. Our results generally agree with literature reports on the importance of particle surface area. For example, Brown et al.^[43] examined the effect of particle size on the inflammatory effects of polystyrene latex spheres (64 nm to 545 nm sized particles) and found significant increases in IL-8 gene expression by A549 cells, a human lung carcinoma cell line with epithelial-like characteristics, after treatment with the smaller diameter particles (and greatest surface area), but not the larger particles. A recent review of in vivo particle instillation studies concluded that lung-deposited particle surface area dose was the toxicologically most relevant dose metric for inhaled, spherical-shaped particles.^[54] The Scr sample also produced the greatest fraction of early apoptotic cells (previous section). This result agrees with in vitro studies directed at understanding the cell death cycle, which have shown that the TNF α induction is one pathway involved in apoptosis caused by ambient PM and carbon nanoparticles.^[55,56]

One might have expected the Bub sample to induce the greatest TNF α cytokine secretion because of its small size. However, each of these treatments used a constant mass dose, and the Bub sample contained a larger fraction of dissolved organic species (Fig. 7a). Thus, the Bub sample included fewer particles compared to the other two samples. These lower numbers of particles likely decreased the interaction between the cdPM and the cells. In addition, the lower TNF α cytokine secretion associated with the Bub sample may also be related to the increased PAH concentration in this sample. In several studies of urban particulate matter, PAHs content was negatively associated with particle-induced production of proinflammatory cytokines and chemokines in THP-1 cells,^[57] in RAW 264.7 cells (a mouse ascites fluid macrophage-like cell line),^[58] and in mouse lungs.^[59,60] Jalava et al.^[58] suggested that PAHs induce immunosuppression and low cytokine production. For in vivo exposure of carbonaceous particles via instillation, Stoeger et al.^[61] found that the fraction of soluble organics was negatively associated proinflammatory response. Overall, the cdPM collection methods can have a large effect on their calculated mass, number and

surface area as well as composition, and these factors all appear to affect subsequent pro-inflammatory responses.

Implications

Understanding the interactions between cellular responses and cdPM physicochemical properties is an important step towards bridging toxicological and epidemiological studies. [57] Although this study focused only on one cell line and did not consider the potentially important contribution of the gas-phase pollutants associated with the combustion, [62] it contributes to our understanding of how particle collection methods can contribute to differences in in vitro responses. Our study suggests that the physicochemical properties of cdPM in the exposure media are critically important when comparing in vitro study results and to eventually translating in vitro results to in vivo models. It also emphasizes the need for more standardized practice for the collection and characterization of cdPM and for the exposure of cdPM to cells, as highlighted by Schmidt et al. [63] Our results showing how cdPM collection methods affect particle size, in particular the loss of the smallest particle sizes presumably through particle aggregation, have implications for investigators using filter-collected and resuspended cdPM in air-liquid interfaces or in exposure chambers.

Conclusion

This study illustrates both the importance of cdPM collection methods on particle physicochemical properties and on biological effects as well as the need for characterizing particles in their exposure media. Specifically, we identified differences in PSD, surface area, and composition caused by different particle collection methods. Bubbling cdPM resulted in the smallest particle size in the suspended phase as well as a bimodal size distribution, similar to that found in the gas phase. The Bub sample also contained substantially more dissolved organic material and lower molecular weight species than the other two samples. However, for the same mass dose, the dissolved species in Bub sample resulted in a lower particle number density and the surface area compared to the Fil and Scr.

These differences in physicochemical properties, in turn, affected oxidative potential, ROS generation, and the inflammatory and apoptotic response. The Bub samples exhibited the greatest DTT consumption and induced the greatest relative ROS production, likely due to the soluble organic content. On the other hand, the Scr samples induced the greatest inflammatory response, as measured by TNF α cytokine production. This increased inflammatory response was likely due to the higher surface area of Scr compared to the Fil and Bub and the inhibition of TNF α cytokine production associated with the higher PAHs content in Fil and Bub. Together these results suggest that particle collection method can affect biological outcomes, that cdPM should be characterized in the media of exposure, and that the effect of particle collection methods on particle properties should be considered part of the experimental planning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of the experimental setup. Post-flame particles were collected: (1) by direct bubbling in DI water, (2) on a 200-nm PTFE filter, and (3) on a water-cooled plate.

Figure 2.

(a) Gas-phase PSD measured with a SMPS for jet-fuel surrogate particles collected at 15 cm above the burner, diluted 1:100. (b) Suspended phase PSD for Bub, Fil, and Scr samples, measured with MALS in lined with the AF4. The particle counts for each sample were normalized with respect to their maximum particle count. Suspended phase PSD in terms of absolute particle counts can be found in supplementary information, Figure 5S.

Figure 3. TEM images of the Bub, Fil, and Scr samples. The images in the first row were taken in STEM mode, and the second row images were taken in TEM mode.

Figure 4. GC-MS chromatograms for the Bub, Fil, and Scr samples. The number indicates the following compounds: 1-naphthalene, 2-biphenylene, 3-hexadecane, 4-fluorene, 5-dibenzofuran, 6-phenanthrene, 7-fluoranthene, 8-pyrene, 9-a derivative of dodecane, 10-chrysene. *lower molecular weight compounds such as phenol, benzaldehyde, and styrene.

Figure 5.

GC-MS chromatogram for 200-nm filtered Bub, Fil, and Scr samples. The number indicates the following compounds: 1-naphthalene, 2-biphenylene, 3-hexadecane, 4-fluorene, 5-dibenzofuran, 6-phenanthrene, 7-fluoranthene, 8-pyrene, 9-derivative of dodecane, 10-chrysene. *lower molecular weight compounds such as phenol, benzaldehyde, and styrene.

Figure 6.

(a) Mass percentage of particle-bound and dissolved organic species in the original samples. DTT consumption rate (pM/μg of sample/min) for: (b) Original samples (Bub, Fil, and Scr); (c) Dissolved organic species (20-nm filtered samples: FBub, FFil, and FScr); (d) Particle-bound species (Bub-FBub, Fil-FFil, and Scr-FScr), after subtraction of the media consumption. Error bars denote SEM (n=3).

Figure 7.

(a) Mass percentage of particle-bound and dissolved organic species in the samples. This panel is the same as 6(a) and is included for reference. (b) ROS response from THP-1 cells to 33 $\mu\text{g/mL}$ cdPM. Error bars denote SEM ($n=6$). (c) Apoptotic response for the three cdPM samples to THP-1 cells at a dose of 33 mg/mL for 24 hr. Error bars denote SEM ($n=3$). (d) TNF α response from THP-1 cells to 33 $\mu\text{g/mL}$ cdPM. Error bars denote SEM ($n=4$). Statistically significant at $**p<0.01$ and $*p<0.05$ for marked pairs.

Table 1.

Physical properties of samples in DI water, prior to addition of media. The numbers in parenthesis indicate the percent standard error.

Sample	Mean rms radius (nm) [*]	Mean hydrodynamic radius (nm) ^{\$}	Number density (#/μg)	Surface area (cm ² /mg sample) [‡]
Bub	48.7 (0.1%)	69.0 (1.1%)	1.01E+8 (0.3%)	3.84 (0.3%)
Fil	57.4 (0.1%)	85.0 (1.4%)	2.33E+8 (0.4%)	12.1 (0.4%)
Scr	85.0 (0.2%)	103 (1.2%)	1.93E+8 (0.7%)	14.1 (0.7%)

* Estimated by ASTRA V 5.3.4 after processing of the MALS signal.

\$ Measured by DLS.

|| Estimated by ASTRA V 5.3.4 after processing of the MALS signal.

‡ Calculated from the rms radius and number density data from ASTRA V 5.3.4. Note that the surface area measurement is the surface area from the particles per mass of sample injected into the AF4 column.

Table 2.

Summary of sample physicochemical properties and biological outcomes relative to the other samples.

Sample property	Relationship to other samples	Biological outcome	Relationship to other samples
Mean particle diameter	Scr > Fil > Bub	Toxicity *	Bub = Fil = Scr
Total particle surface area	Scr > Fil > Bub	ROS	Bub > Fil > Scr
Total particle count	Fil > Scr > Bub	TNF α	Scr > Bub and Fil
Dissolved organic content	Bub > Fil > Scr	Apoptosis	Scr > Bub and Fil

*At a mass dose of 33 $\mu\text{g/mL}$.