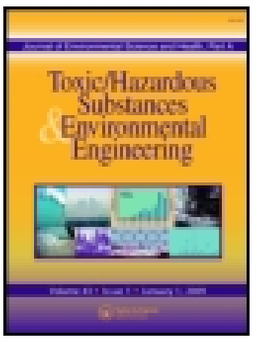

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Properties and cellular effects of particulate matter from direct emissions and ambient sources

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ABSTRACT

The pollution of particulate matter (PM) is of great concern in China and many other developing countries. It is generally recognized that the toxicity of PM is source and property dependent. However, the relationship between PM properties and toxicity is still not well understood. In this study, PM samples from direct emissions of wood, straw, coal, diesel combustion, cigarette smoking and ambient air were collected and characterized for their physicochemical properties. Their expression of intracellular reactive oxygen species (ROS) and levels of inflammatory cytokines (i.e., tumor necrosis factor-α (TNF-α)) was measured using a RAW264.7 cell model. Our results demonstrated that the properties of the samples from different origins exhibited remarkable differences. Significant increases in ROS were observed when the cells were exposed to PMs from biomass origins, including wood, straw and cigarettes, while increases in TNF-α were found for all the samples, particularly those from ambient air. The most important factor associated with ROS generation was the presence of water-soluble organic carbon, which was extremely abundant in the samples that directly resulted from biomass combustion. Metals, endotoxins and PM size were the most important properties associated with increases in TNF-α expression levels. The association of the origins of PM particles and physicochemical properties with cytotoxic properties is illustrated using a cluster analysis.

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in vitro toxicity test; particulate matter; physicochemical properties; ROS; TNF-α

Introduction

Among various concerning air pollutants, particulate matter (PM) is at the top of the priority pollutant list in China because of its severe contaminations and well-known adverse health impacts.[1–3] Several long-lasting heavy air pollution episodes affecting the entirety of eastern China in early 2013 gained the attention of both government and public agencies.[4] Although a series of efforts were made in recent years,[5] the average daily concentrations of $PM_{2.5}$ in many cities were still several times higher than the national standard of 75 $\mu g/m^3$.[6] It appears that the problem will not be solved soon. As a complex mixture, ambient PM originates from both primary emissions and secondary formation mechanisms.[7] The primary PM in China is mainly a result of anthropogenic activities, including energy production, industrial activities, motor vehicles and residential burning of coal and biomass fuels.[8] In addition, PM is also produced from important sources such as spring sandstorms and resuspension of local soil.[9,10]

The physical properties and chemical compositions of PM from different sources vary extensively. In fact, the physicochemical properties of PM are often used as an indicator for source apportionment in receptor modeling.[11] For example, typical PMs emitted from diesel engines are agglomerates of spherical primary particles with diameters from 0.015 to 0.040 μm,[12] while the dominant size fractions of PMs are less than 2.1 μm for those released as a result of residential wood burning; similarly, particles generated from crop residue combustion have particles with diameters between 1.1 and 3.3 μm.[13] Emission factors of particulate-phase polycyclic aromatic hydrocarbons (PAHs) from various residential solid fuels vary by two orders of magnitude.[14] Among others, fuel properties, including moisture and ash content and combustion efficiency, are important factors that affect the physicochemical properties of the emitted PM.[15] By comparison, emission factors of particulate-phase PAHs from diesel combustion are significantly lower than those resulting from the combustion of residential coal and biomass fuels.[16]

Exposure to PM can cause a variety of adverse health effects, including lower respiratory illness, chronic obstructive pulmonary disease, cerebrovascular disease and lung cancer.[17] Among the various methods that are used to assess the toxicity of PM, *in vitro* tests that use cell lines are widely adopted as fast-screening procedures.[18–20] This method is also useful for investigating toxicity mechanisms at the cellular level.[21] For

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example, a RAW264.7 macrophage cell line was used to investigate the toxic mechanisms of PM generated under different biomass combustion conditions.[22] It was found that PM emitted during smoldering and intermediate combustion periods had relatively small impacts on cellular metabolic activity but caused a relatively high amount of DNA damage compared to particles emitted through efficient combustion.[22] The effects of ambient PM on the secretion of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) from cultured alveolar macrophages were studied, and a dose-dependent effect on the inflammatory macrophages was observed.[23] The cell line RAW264.7 has also been adopted to investigate the cell toxicity mechanism of ambient PM in Los Angeles.[24]

The observed differences in the toxic effects of PM emitted from different sources or locations are believed to have been a result of variations in physicochemical properties. For example, the *in vitro* formation of intracellular reactive oxygen species (ROS) in three cell lines (i.e., CaCo-2, MDCK and RAW264.7) in the presence of particles collected in Germany was found to be positively correlated with particle sizes, and the highest rise in ROS was provoked by the two smallest PMs.[25] Size dependence of cytotoxicity was also reported for PM samples collected in Mexico and Beijing.[20,26] In a study testing the *in vitro* inflammatory and cellular metabolic activity of PM collected from Helsinki, Finland, using the macrophage cell line RAW264.7, it was demonstrated that the toxicological responses depended on size ranges, components and doses of the PM.[27] The particle-induced inflammatory responses were positively correlated with dust from road traffic in coarse particles and were negatively associated with PAHs and potassium in fine particles.[27]

The objective of this study was to test the differences in both physicochemical properties and cellular effects of PMs from different origins. To this end, seven PMs were collected either directly from emissions of wood, crop residue, coal, diesel combustion and cigarette smoking or from ambient air in Beijing and Wuwei, China. The samples were characterized for a number of physicochemical properties and tested for their toxicological activity *in vitro* using a RAW264.7 cell line. The production of inflammatory cytokines was measured, and the generation of TNF-α and the level of ROS were used as endpoints. The relationship between the physicochemical properties of the PMs and their *in vitro* toxicities is discussed.

Methodology

Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and trypsin–EDTA were purchased from GIBCO, Waltham, MA, USA. Dihydroethidium (DHE), dithiothreitol (DTT), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), penicillin and streptomycin were obtained from Sigma-Aldrich, St. Louis, MO, USA. Surrogates (i.e., 2-fluoro-1,1′-biphenyl and *p*-terphenyl-d_{14}) and internal standards (i.e., naphthalene-d_8, acenaphthene-d_{10}, anthracene-d_{10}, chrysene-d_{12} and perylene-d_{12}) for PAH analysis were purchased from J&K Chemicals, Sunnyvale, CA, USA, while the PAH standards were purchased from Chem Service, West Chester, PA, USA. Other reagents were purchased from Beijing Reagent, China. The glassware, glass fiber filters, silica gel as well as aluminum oxide used for the experiments were baked at 450°C for 4 h prior to use, and anhydrous sodium sulfate was heated at 650°C for 6 h to remove moisture from the reagents. To be reactivated, silica gel and aluminum oxide were baked at 130°C for another 16 h immediately before use.

Sample collection

PM_{10} samples were collected from five direct emission sources in China: (1) wood (pine), (2) crop residue (cornstalk), (3) coal (anthracite from Fujian), (4) cigarette (mainstream of smoke produced from a low-quality brand of Hongqiqu) and (5) diesel from a diesel engine (Changchai, ZS1105, installed in a tractor); and two urban ambient sources: (6) Beijing and (7) Wuwei. The first three were burned in a cast iron stove in a laboratory chamber. The PM samples were collected on Teflon filters using sampling pumps operated at a flow rate of 4 L/min (source samples, Buck Libra Plus LP-5, Orlando, FL, USA) or 100 L/min (ambient samples, PM10-PUF-300, Guangzhou, China). The PM samples collected on the filters were ultrasonically (50 W, KQ-50, Kun Shan, China) extracted with 30 mL of methanol for 5 min and concentrated to 2.0 mg/mL in methanol. The suspensions were concentrated to 1 mL using a rotary evaporator at 37°C, freeze dried (EYELA-FDU-830, Tokyo Rikakikai, Tokyo, Japan), and resuspended in water as the stock solution with 100 μg/mL streptomycin sulfate and 100 U/mL penicillin, which was stored at −80°C prior to use. It is well recognized that the possible effects of the procedure on PM size and composition could not be avoided. Therefore, a control without PM was prepared using the exact same procedure.

Characterization of the PM samples

A PM suspension of 400 μg/mL in DMEM with 10% FBS was used for zeta potential and size distribution measurements (ZS90, Malvern, UK). An aqueous PM suspension at 400 μg/mL was shaken for 16 h and filtered. The filtrate was analyzed for water-soluble organic carbon (WSOC) matter using a total carbon analyzer (TOC-5000A, Shimadzu, Japan), and the contents of NO_3^- were measured using an ion chromatograph (792 Basic IC, Switzerland). Inductively coupled plasma-atomic emission spectrometry (Optima 3300DV, PerkinElmer, Waltham, MA, USA) was used for metal determination after the samples were digested using a mixture of HNO_3 and $HClO_4$ following the method reported by Lee et al.[28] The intrinsic oxidant potentials were determined using a DTT assay with a cell-free system, as described in the literature.[29] Endotoxin levels were measured using Limulus amebocyte lysate assay kits (Chinese Horseshoe Crab Reagent Manufactory, China). PAH levels in the exposure media spiked with PM were measured. In brief, the samples were extracted using a microwave accelerated reaction system (CEM, New York, NY, USA), purified using silica/alumina columns and analyzed using a gas chromatograph (Agilent 6890, Santa Clara, CA, USA) coupled with a DB-5MS capillary column and a mass spectrometer (Agilent 5973). The 19 parent PAHs that were quantified include acenaphthene (ACE), acenaphthylene (ACY), fluorene (FLO), phenanthrene

(PHE), anthracene (ANT), fluoranthene (FLA), pyrene (PYR), benzo(*a*)anthracene (BaA), chrysene (CHR), benzo(*b*)fluoranthene (BbF), benzo(*k*)fluoranthene (BkF), benzo(*a*)pyrene (BaP), diben(*a,h*)anthracene (DahA), indeno(1,2,3-*cd*)pyrene (IcdP), benzo(*g,h,i*)perylene (BghiP), benzo(*e*)pyrene (BeP), perylene (PER), retene (RET) and cyclopenta(*c,d*)pyrene (CcdP). For the metals and PAHs, procedure blanks were measured with each batch of samples and were subtracted from the results. For the PAHs, the detection limits were 0.53–1.32 ng/g, and the recovery rates ranged from 69% to 115% for the standard spiked solutions and from 80% to 103% for the surrogates (i.e., 2-fluoro-1,1′-biphenyl and *p*-terphenyl-d_{14}). The recovery rates for metals in the reference material (NIST 1648a) were 106%, 100%, 101%, 95%, 108%, 104%, 96% and 87% for Cd, Cu, Pb, V, Zn, Ca, Fe and Mg, respectively.

Cell cultivation and exposure

The murine monocyte–macrophage cell line RAW264.7 (Cell Resource Center, IBMS, China) was cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C (5% CO_2, Sanyo, Japan). Wells (12-well plates) were seeded with 2.5×10^5 cells/well 24 h before exposure, during which the cells grew up to 70% confluence. Particle stock suspensions were sonicated for 3 min, vigorously vortexed and diluted to the final concentrations of 50, 100, 200 or 400 μg/mL based on the results of a preliminary experiment. RAW264.7 cells were exposed to PM suspensions (100 μL each) along with the control. It should be pointed out that components in soluble or solid phases were not able to be distinguished in such a procedure. The plates were incubated at 37°C in a CO_2 incubator (5% CO_2, Sanyo) for 16 h. The culture medium was collected and centrifuged at 1000*g* for 10 min after incubation. The supernatants were immediately stored at −80°C for further TNF-α analysis, and the rest of the cells were labeled and used for ROS detection.

Bioassay

Oxidative stress and inflammatory effects were characterized by ROS generation and the release of TNF-α. The intracellular generation of ROS was analyzed by flow cytometry using DHE staining.[30] Twenty-one hours after the addition of the PM, the cells were incubated with 20 μM DHE for 10 min at 37°C and rinsed twice with DMEM to remove the excess fluorescent probe. The trypsinized, suspended and collected cells were immediately analyzed by flow cytometry (Becton Dickinson, USA) at wavelengths of 488 and 630 nm for excitation and emission, respectively. The generation of TNF-α in the cell culture media was measured using an enzyme-linked immunoabsorbant assay (ELISA) using a commercially available kit (Wuhan Boster, China) and a microplate reader at 450 nm according to the manufacturer's instructions.

Data analysis

The measurements were conducted in duplicates for the WSOC and PAHs; triplicates for endotoxin, ROS and TNF-α; and hexaplicates for DTT. The sample quantities were insufficient for duplicate measurements for PM size, zeta potential, NO_3^- and metals. For the samples with duplicate measurements, the results are presented as mean ± standard deviation. Analyses of variance, *t*-tests, non-parametric correlation analyses and hierarchical cluster analyses were performed using SPSS v. 13.0 (SPSS Inc., Chicago, IL, USA). A significance level of 0.05 was adopted. The between-groups linkage cluster method and squared Euclidean distance were applied to the cluster analyses after normalization.

Results

Physicochemical properties

The measured sizes, surface zeta potentials, concentrations of WSOC, NO_3^-, endotoxin of the PMs and DTT-based redox activities in the exposure medium loaded with the PMs are listed in Table 1.

Of seven samples tested, the median diameters of the PMs from ambient air were the smallest due to the contribution of secondary aerosols (Table 1). According to the results of a previous study on ambient PM in Beijing, the median diameters varied from 0.42 to 0.62 μm,[19] similar to a value of 0.9 μm that was observed in this study. The median diameters of the samples resulting from all the biomass combustion sources were larger than those from fossil fuel combustion, which were in agreement with values reported in the literature. It has been recently found that the size distribution of particles emitted from residential wood and straw burning peaked at 0.7–1.1 μm,[13,15] while the peak size of PM from a four-stroke diesel engine was approximately 0.09–0.18 μm.[31] The measured zeta potentials varied from −40 to 3 mV. The values for the ambient air samples were similar to those reported previously.[19,20] The negative charge of ambient PMs can be explained by proton dissociation and sorption of negatively charged proteins.[32] Significant differences in zeta potentials

Table 1. Median diameters, surface zeta potentials, WSOC, NO_3^-, endotoxin and DTT of the PM samples or exposure media spiked with the PM samples.

Sample source	Cigarette	Wood	Straw	Coal	Diesel	Beijing	Wuwei
Median diameter*, μm	30.0	17.5	15.6	3.6	5.5	0.9	2.0
Zeta potential*, mV	−22	3	−4	−47	−40	−30	−14
WSOC, mgC/g	220 ± 0.84	250 ± 1.22	130 ± 0.38	11 ± 0.05	32 ± 0.22	3.9 ± 0.11	1.5 ± 0.29
NO_3^-*, mg/g	3.1	3.2	5.6	2.9	8.4	34.6	11.2
Endotoxin, eu/mg	1.5 ± 0.02	0.81 ± 0.02	0.88 ± 0.01	1.2 ± 0.02	1.3 ± 0.02	1.2 ± 0.05	8.3 ± 1.27
DTT redox ability, nmol/(min μg)	0.06 ± 0.004	0.06 ± 0.006	0.08 ± 0.002	0.04 ± 0.003	0.13 ± 0.004	0.06 ± 0.001	0.04 ± 0.003

*Quantities of the samples were insufficient for duplicate measurements.

between the PMs directly emitted from biomass and fossil fuel combustion were observed in this study.

WSOC in PM originates from either primary emission or secondary formation. Although significant contributions of secondary WSOC have often been reported in the literature,[33,34] WSOC contents in PM resulting from direct emissions by cigarette, wood and straw burning (13–25%) were 1 or 2 orders of magnitude higher than those emitted from fossil fuel combustion and ambient air, respectively. This is similar to a 20% increase that was reported for emissions generated from rubber wood and rice husk combustion.[35] WSOC contents in the PM of ambient air were similar to those in the PM previously collected in Beijing.[19] The secondary aerosols in urban air are often related to the transportation sector.[34,36] The larger number of motor vehicles in Beijing can explain the relatively high WSOC levels in the PM samples collected from Beijing compared with those collected from Wuwei. However, mineral dust is likely an important composition of the PM originating from Wuwei, which is located in the semiarid region of West China.

The NO_3^- contents in the two ambient samples were higher than those of the others due to exhausts from on-road motor vehicles in the cities. This is particularly true for Beijing where there are more than five million vehicles.[37] High-temperature reactions also cause relatively high NO_3^- concentrations in PM emitted through diesel exhaust. Although the levels of endotoxins generally agreed with those previously reported for PM samples obtained from Beijing (3.0–17 EU/mg),[20] a moderate difference ($P < 0.05$) in the measured endotoxin concentrations among the samples, which was relatively small compared with most other properties, could not be explained based on the information regarding the PM sources. The average DTT redox abilities were similar to those reported for the PM collected from Beijing using the same cultural media.[19,20] Similar to endotoxin, there was no significant difference among the samples, except for the relatively high value for the PM emitted from diesel exhaust ($P < 0.05$).

Metals

The metal concentrations are listed in Table 2. Although K is generally referred as a marker of biomass burning because it is ubiquitous in the cytoplasm of plants,[38] relatively high K contents were only found in the PM emitted through straw burning ($P < 0.05$) and not wood. Relatively high concentrations of Ca, Fe, Mn and most trace elements, including all chalcophile elements (i.e., Cd, Cu, Pb and Zn), together with relatively low concentrations of WSOC in the sample from Wuwei, confirmed that the main component of the PM was desert dust. For trace elements, the contents in the PM from fossil fuels, particularly coal, were generally higher than those from biomass burning, including cigarettes ($P < 0.05$). For some metals such as V and Ni, the differences were as high as 1 order of magnitude. High levels of Zn and Pb in the PM from cities may have been caused by traffic and other anthropogenic sources.

Table 2. Metal concentrations of the PM samples.

	PM samples						
Metals	Cigarette	Wood	Straw	Coal	Diesel	Beijing	Wuwei
K, mg/g	12.9	2.1	20.3	11.1	10.5	1.5	12.1
Na, mg/g	16.9	2.1	2.2	12.6	13.6	1.3	11.6
Ca, mg/g	11.8	1.5	1.5	14.3	19.9	4.5	42.5
Mg, mg/g	0.35	0.04	0.04	0.31	0.29	0.03	0.29
Fe, mg/g	1.21	0.09	0.14	2.04	1.71	0.83	17.3
Mn, mg/g	0.018	0.003	0.005	0.076	0.051	0.040	0.448
Cr, mg/g	0.52	0.26	0.29	1.28	1.84	0.19	0.03
Cu, μg/g	16.0	3.8	4.1	31.7	30.8	19.5	90.5
Cd, μg/g	1.49	0.22	3.31	1.22	0.81	1.78	2.98
Pb, μg/g	6.7	6.3	12.0	85.2	39.3	69.9	98.0
Zn, μg/g	184.1	34.6	34.1	195.1	178.2	235.8	378.1
Ni, μg/g	6.7	1.5	1.2	16.2	9.6	2.9	43.7
Co, μg/g	67.7	8.1	8.3	82.4	66.9	5.0	77.0
V, μg/g	0.5	0.3	0.2	10.6	5.5	3.4	22.5

Polycyclic aromatic hydrocarbons

The measured PAH concentrations in the culture media are listed in Table 3 as means and standard deviations.

Significant differences can be observed among the samples from different sources ($P < 0.05$), and the highest PAH concentrations were associated with PMs emitted through cigarette smoking and coal burning. It is interesting to note that the PAHs released into the cultural media were not correlated with WSOC. The WSOC concentrations in PM from three biomass origins were significantly higher than those generated by other sources, whereas no such difference was observed for the PAHs in the culture media. Of the individual compounds, the concentrations of PAHs with small molecular sizes were much higher than those with large molecular sizes due to the relatively high solubility of smaller PAHs. The highest concentrations of most two- to three-ring compounds were found in the PM from cigarettes, as often reported in the literature.[39] For the PM emitted by coal combustion, the concentrations of high-molecular-weight PAHs from BaA to CcdP were more than 2 orders of magnitude higher than those of the others, and similar results were found for the PM emitted by residential coal combustion.[14]

Reactive oxygen species

Oxidative stress induced by excessive ROS is a major mechanism responsible for the adverse health effects of PM.[40] The dose responses of the RAW264.7 cell line to the PM obtained from different origins at three exposure concentrations are shown in Fig. 1. Significant differences were observed for the PM sources and concentrations, and these results were confirmed by two-way analyses of variance ($P < 0.05$). Significant dose-dependent increases in intracellular ROS were observed for the PM samples that had a range of 100–400 μg/mL ($P > 0.05$), except for samples obtained from cigarette smoking and ambient air in Beijing. In a previous study that used a J774A.1 cell line to test the cytotoxicity of PM samples collected during different seasons in Beijing, a significant dose–response relationship was also observed.[19] It was very interesting to see the significant differences in the measured ROS levels between the two PM categories. For all the tests that used samples from biomass origins, including cigarettes, wood and straw, the PM-induced redox activities were significantly higher than that of the control, indicating a strong influence on the generation of ROS. The generation of exogenous ROS by pollutants or tobacco smoke has been commonly reported.[41,42] However, although there were significant dose–response

Table 3. The measured PAH concentrations in the exposure media loaded with various PM samples (μg/mL).

	PM samples						
PAHs	Cigarette	Wood	Straw	Coal	Diesel	Beijing	Wuwei
ACE	16.00 ± 9.51	0.77 ± 0.04	0.79 ± 0.04	23.59 ± 12.7	5.26 ± 0.55	0.50 ± 0.02	7.91 ± 1.57
ACY	2.58 ± 1.84	0.15 ± 0.01	0.14 ± 0.02	1.06 ± 0.10	0.86 ± 0.12	0.10 ± 0.01	1.13 ± 0.03
FLO	60.70 ± 40.5	3.26 ± 0.31	3.07 ± 0.31	24.46 ± 0.27	20.39 ± 0.40	2.62 ± 0.97	25.60 ± 1.11
PHE	95.61 ± 62.3	4.45 ± 0.49	5.02 ± 0.15	34.81 ± 2.96	30.97 ± 0.49	4.27 ± 2.01	38.72 ± 2.10
ANT	139.4 ± 90.7	0.49 ± 4.16	3.87 ± 0.07	3.88 ± 0.07	3.83 ± 0.57	0.81 ± 0.69	31.26 ± 38.7
FLA	10.28 ± 6.54	0.58 ± 0.01	0.62 ± 0.00	7.21 ± 0.63	6.36 ± 1.14	0.74 ± 0.11	6.25 ± 1.14
PYR	86.30 ± 59.2	4.06 ± 0.12	3.97 ± 0.20	34.78 ± 3.34	45.14 ± 5.77	3.13 ± 0.25	38.95 ± 3.97
BaA	4.93 ± 4.86	0.21 ± 0.01	0.15 ± 0.11	11.42 ± 1.40	7.79 ± 2.28	0.52 ± 0.04	4.05 ± 0.14
CHR	4.77 ± 4.86	0.25 ± 0.01	0.23 ± 0.01	21.81 ± 3.36	5.27 ± 1.55	1.11 ± 0.07	7.17 ± 1.18
BbF	N.D.	0.02 ± 0.00	N.D.	19.51 ± 2.61	0.14 ± 0.06	1.02 ± 0.58	3.62 ± 0.14
BkF	N.D.	0.04 ± 0.03	0.02 ± 0.06	14.17 ± 1.89	0.20 ± 0.11	0.82 ± 0.31	2.40 ± 1.35
BaP	N.D.	N.D.	N.D.	7.16 ± 0.59	N.D.	0.24 ± 0.01	0.56 ± 0.80
DahA	N.D.	N.D.	N.D.	1.05 ± 0.39	N.D.	N.D.	N.D.
IcdP	N.D.	N.D.	N.D.	1.73 ± 0.27	N.D.	0.16 ± 0.01	0.35 ± 0.50
BghiP	2.36 ± 2.04	0.14 ± 0.00	0.10 ± 0.03	5.77 ± 1.07	0.80 ± 0.21	0.22 ± 0.00	1.87 ± 0.50
BeP	0.61 ± 0.86	0.07 ± 0.04	0.03 ± 0.01	14.76 ± 2.09	0.59 ± 0.08	0.42 ± 0.00	2.74 ± 1.13
PER	N.D.	0.02 ± 0.03	0.02 ± 0.03	0.55 ± 0.13	N.D.	0.03 ± 0.04	0.17 ± 0.24
RET	16.85 ± 11.3	6.95 ± 2.12	8.38 ± 8.70	67.65 ± 9.53	10.23 ± 1.66	0.73 ± 0.15	22.94 ± 21.5
CcdP	N.D.	N.D.	N.D.	0.85 ± 0.34	0.41 ± 0.57	0.19 ± 0.02	0.62 ± 0.88

N.D.: under detection limits.

relationships in most cases, the generation of ROS induced by the PM originating from fossil fuel combustion (i.e., coal and diesel) or ambient air (i.e., from Beijing and Wuwei) were not significantly different from that of the control.

Tumor necrosis factor-α

As a cell signaling protein (cytokine) secreted predominantly by monocytes and macrophages, TNF-α is involved in local and systemic inflammation reactions.[43] Figure 2 shows the measured TNF-α levels for RAW264.7 cells exposed to the seven PM samples. Clear dose-dependent responses were observed in all cases ($P < 0.05$). TNF-α expression was very low in the control compared with those exposed to PM. The responses of the cells varied among the PMs that originated from different sources. For example, at a PM concentration of 200 μg/mL, the measured TNF-α expressions were in the following order: Beijing > Wuwei > cigarette = coal = diesel > straw > wood ($\alpha = 0.05$). This was almost a reversed order as that of ROS, indicating that the factors affecting ROS and TNF-α were rather different. At relatively low PM levels of 50 and 100 μg/mL, similar, but less obvious trends could be observed. In general, the measured TNF-α levels for cells exposed to the PM obtained from the two ambient sources were several times higher than those incubated with the PM that resulted from direct emissions. In a recent study, TNF-α expression, together with other inflammatory responses to aqueous extracts of PM collected near a major Los Angeles freeway, was measured using a THP-1 cell model.[44] A significant increase in TNF-α has been observed, and the induction of proinflammatory protein expression could be attenuated by kinase inhibitors or polymyxin B, indicating the roles of protein kinases and endotoxin.[44] Using PM samples of different size ranges or those collected during different seasons in Beijing, the release of TNF-α from J774A.1 cells and its dose dependence were also reported.[19,20]

Discussion

Reactive oxygen species

The results of our study revealed that the amounts of ROS induced by PM from biomass origins were much higher than those from fossil combustion or ambient air. The major

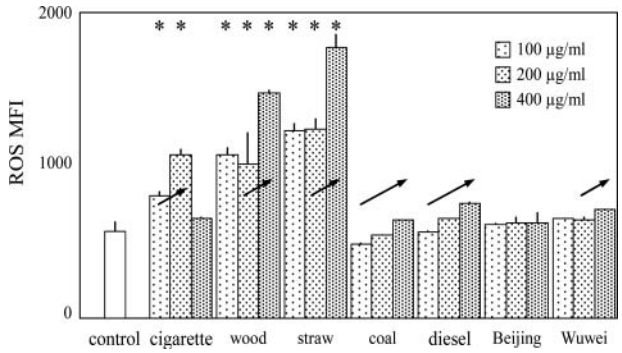

Figure 1. ROS production in RAW264.7 cells induced by PM from different origins at different concentrations. The results are presented as means and standard deviations. Significant differences in the measured mean fluorescence intensities (MFIs) between the treatments and the control are marked with "*", while significant dose responses are labeled with arrows ($P < 0.05$).

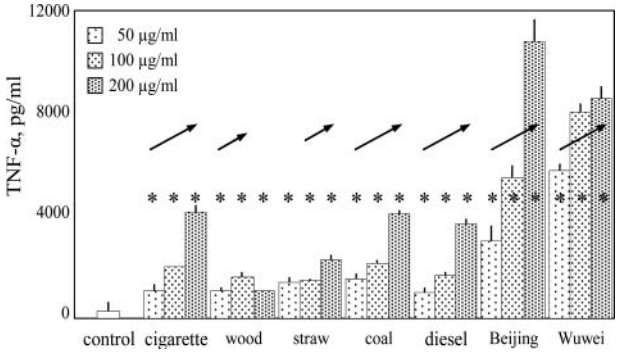

Figure 2. The measured TNF-α expression by RAW264.7 cells exposed to PM from different origins at different concentrations. The results are presented as means and standard deviations. Significant differences in the measured TNF-α levels between the treatments and the control are marked with "*", while significant dose responses are labeled with arrows ($P < 0.05$).

differences between the two categories were the amount of WSOC, particle sizes and zeta potentials.

The association of aerosol-induced intracellular ROS generation with WSOC content has been reported in the literature.[45–47] For instance, by investigating the biological oxidative potential of size-segregated PM samples collected from different urban areas of the world, a significant correlation between ROS and WSOC was revealed.[46] In another study, PM samples collected from different environmental settings in the southeast region of the United States were tested to identify major ROS-associated emission sources, and a similar correlation between ROS generation potential and WSOC contents was revealed.[47] It was also noted that the correlation between ROS generation and WSOC content was observed mainly for PM that underwent secondary formation in the summer or the PM generated from biomass combustion in the winter.[47]

Unlike most other studies that used ambient air aerosols, five out of the seven PM samples that were collected directly from combustion sources in this study and the WSOC contents of these samples, especially those from biomass combustion, were much higher than those of samples collected from ambient air. Freshly emitted PM from biomass combustion with very high WSOC levels led to high ROS activities in the tested cells. Because residential energy in developing countries, including China and India, is dominated by biomass fuels,[48] the relative contribution of organic carbon to PM composition in developing countries has been shown to be higher than that in developed countries.[8] It appears that this is the major reason why PM collected from developing countries is often more ROS active than samples collected from developed countries.[46] The significant contributions of biomass combustion to both WSOC and ROS of ambient PM have been confirmed previously.[45,47,49] For example, in a study using $PM_{2.5}$ samples collected in Baghdad, a moderate correlation between ROS and WSOC from biomass open combustion was identified.[45] It will be very interesting in the future to identify the major WSOC components that are responsible for ROS activity.

Based on our measurements, ROS and PM median size showed a positive correlation ($P < 0.05$). However, the relationship between the two has been shown to be controversial in the literature. For instance, a similar positive correlation was found between particle size and ROS generated from both A549 and J774A.1 cells for PM collected in Beijing during different seasons,[19] while a negative correlation between ROS and PM size has been reported in some other studies.[47] It is likely that the positive correlation observed in this study was not a casual effect, and the PM from biomass combustion with high amounts of WSOC happens to be large in size. It is also possible that coarse PM readily settles due to its higher mass and then has more opportunities to come in contact with cells.[20]

Many other PM properties, including transition metals, PAHs and DTT activity, have been reported to be associated with ROS.[19,47,50] For example, correlations between ROS and water-soluble transition metals, including Fe, Ni, Cu, Cr, Mn, Zn and V, have been reported, and it is believed that both traffic emissions and secondary organic aerosol formation are the major sources of PM-induced oxidative potentials.[46] However, no correlation between ROS and the concentration of any individual metal or PAH (dissolved in the cultural media) was identified in this study. Their effects could have been concealed by the strong influence of WSOC and may have been difficult to statistically quantify due to the small sample size. However, indirect effects cannot be ruled out for the associations reported in the literature.

Tumor necrosis factor-α

Although all the samples collected in this study had significant potentials for driving pulmonary inflammation, the levels of TNF-α expression showed the highest responses to the two samples collected from urban ambient air. Among all the physicochemical properties measured in this study, TNF-α expression had a significant positive correlation with NO_3^-, endotoxin and a number of metals, including Fe, Mn, Cu, Pb, Zn, Ni, Co and V, and was negatively correlated with particle size and WSOC contents ($P < 0.05$). No dependencies were observed for PAHs or for the total or individual compound concentrations.

For all the samples at various exposure concentrations, the highest value of TNF-α expression occurred for the treatment using PM from ambient air in Beijing at a concentration of 200 μg/mL. Additionally, the PM from Beijing led to the steepest dose–response relationship. Comparing these observations with those of the other samples, the most profound difference of the PM from Beijing was that it had the highest NO_3^- concentration, which was 3–4 times higher than those collected from Wuwei and diesel engine exhaust and an order of magnitude higher than PM resulting from biomass and coal combustion. Although NO_3^- is a major air pollutant in urban areas, which is predominantly emitted by motor vehicles, it was not often measured in the *in vitro* cytotoxicity studies. Among a few tests that included NO_3^-, a negative correlation was reported between NO_3^- and inflammatory activities in mice.[51] To date, no conclusions have been made on the impact of NO_3^-. The observations noted in this study could have been an indirect correlation, and the influence of NO_3^- should be tested by suppressing NO_3^- in the system in future studies.

Endotoxin is often found to be an important property associated with inflammatory effects. For example, it was found that the inflammatory response induced by PM from a Los Angeles freeway could be reduced by pretreating the sample with polymyxin B, which confirmed its role.[44] By exposing RAW264.7 cells to PM samples from eight sites in the Netherlands, it was demonstrated that the release of proinflammatory markers, including TNF-α, was associated with the endotoxin levels of the coarse samples.[52] In another study, it was shown that PM that had the highest endotoxin content could elicit the greatest proinflammatory response in mice, including increases in the number of IL-6 and MIP-2 cytokines and TNF-α.[53] Similar results were found in our study; the PM from Wuwei had the highest endotoxin contents and could induce a high expression level of TNF-α by the cells.

The correlation between TNF-α expression and metal concentrations found in this study is generally in agreement with those reported in the literature. A number of metals, including soil-derived constituents of Ca, Al, Fe and Si and transition

metals such as Ni and V in PM from European countries, have shown positive correlations with fine PM-induced inflammatory activity.[51] In another study, it was found that a metal-induced pulmonary response could be substantially diminished after the soluble metals were removed by chelation.[54] In addition to endotoxin, the highest concentrations of most metals in the PM from Wuwei are likely another reason for the high TNF-α expression levels.

A negative correlation between TNF-α and size was also revealed in this study. Coarse PMs have been often found to be more effective in inducing cellular inflammation in the literature.[55] For instance, in a study on acute pulmonary injury in rats by ambient PM collected from different sites in Mexico City, it was found that PM_{10} had a more pronounced influence than $PM_{2.5}$ on the elevation of inflammation biomarkers.[56] Although PAHs have been occasionally reported to be responsible for inflammatory responses of PM,[57] no such association was observed in this study.

Association between PM origins and properties

To illustrate the link between the sample origins and their properties (both physicochemical and cytotoxic), a cluster analysis was conducted for the seven PM samples from different origins based on all the properties that were measured in this study. The results are shown in Fig. 3 as two dendrograms for the origins (top) and properties (bottom). The seven PM samples were clearly classified into three categories (i.e., biomass, fossil fuels and ambient air origins), which exhibited significantly different physicochemical properties and cytotoxicity.

For the property clustering, PAHs, metals, other properties and the two endpoints were distinguished to a great extent. The first cluster of the origin dendrogram (biomass) corresponded to the last cluster of the property dendrogram from K to zeta potentials. The most distinguished feature of the PM from biomass combustion was the very high WSOC content, relatively large size and strong ROS induction ability of the samples. Relatively high K concentrations were also an important indicator of PM with biomass origins. The ambient PM (the last category of the origin dendrogram) could be linked to the middle cluster from NO_3^- to Cr in the property dendrogram, which is dominated by metals, endotoxin and TNF-α generation. Finally, the cluster from ACY to BaA in the property dendrogram, which consists mostly of PAH compounds, showed no influence on either endpoint and had no linkage to any origin cluster.

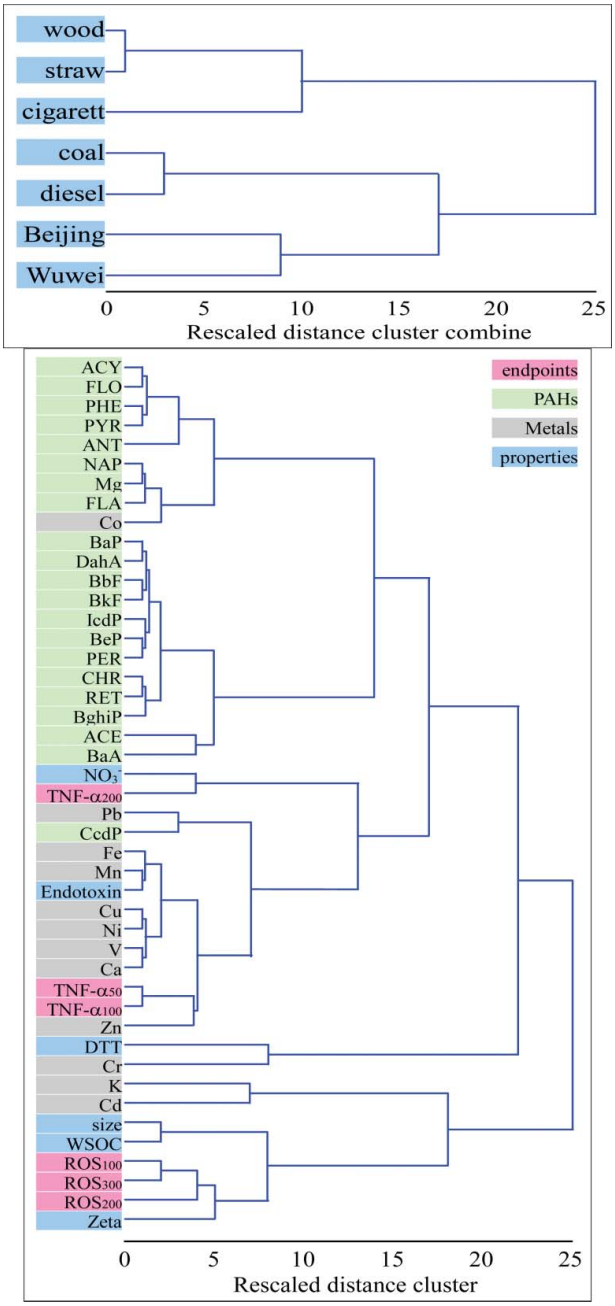

Figure 3. Dendrograms using average linkages for the seven PM samples from different origins (top) and all the parameters, including the two cytotoxic endpoints (ROS and TNF-α) and all the physicochemical properties measured in this study (bottom). For the latter, the variables were classified into four categories: cytotoxicity endpoints, PAHs, metals and all other physicochemical properties. All data were standardized before performing the cluster analysis.

Conclusion

PM samples from different origins were very different in physicochemical properties as well as cytotoxicity for the cells tested (RAW264.7). The most important finding is that the PMs from biomass origins, including those from wood burning, straw burning and cigarette smoking, could induce much stronger oxidative stress in terms of ROS generation in comparison with PMs from coal, diesel and ambient air. Although all PMs can induce the release of TNF-α, the influence of PMs from ambient air was the strongest. The most important factor associated with ROS generation was WSOC, which was extremely abundant in the PMs of biomass origin. Metals, endotoxins and PM size were the most important properties associated with increases in TNF-α expression levels.

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