

Characterization and Inflammatory Potential of sub-10nm Particles from Gas Cooking Appliances

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Combustion generated ultrafine particles are believed to have an effect on human health. Their presence in the atmosphere is mainly attributed to outdoor sources, but they may also form indoor. Gas cooking is a widely diffused indoor activity commonly considered environmentally clean, and without emissions of particulate matter. However, even bluish flames of natural gas may produce considerable number concentrations of sub-10nm particles if operating conditions deviate from stoichiometry and mixing at atomic level. These particles negligibly account for particulate mass but, due to their very low sizes, they can deposit far inside the airways and on skin and potentially reach target organs being dangerous although present in low mass concentrations. We have characterized the exhausts of a domestic cooktop burner measuring stable compounds, gas-phase aromatic compounds and particulate matter and collected nanoparticles for *in vitro* toxicological studies and for the analysis of their possible inflammatory effects. Combustion exhausts, including polycyclic aromatic hydrocarbons (PAH) and nanoparticles, have been sampled above a mid-range cooktop burner fed with network natural gas. Tests have been performed in a free flame and by putting a pot on the burner in order to simulate operating conditions closer to those of the real life. Speciation of PAHs and the distribution of the particles generated during combustion has been measured. Results of measurements show that the cooktop burner flames produce and emit low concentrations of PAHs and huge number concentrations of sub-10nm particles. Tests on cell viability performed with crystal violet assay shows no significant reduction in cell number after 24h of treatment, both with nanoparticles collected in a “free flame” and in the operating conditions with “a pot on the fire”. It is interesting to note a little positive effect in increasing cell number (+20%) at the lowest concentration. No relevant overexpression or downregulation is noted on the secretion of the 27Plex Panel of Human Cytokine, performed with Bio-Plex 200 system.

1. Introduction

Gas cooking is the most widely and frequently used indoor combustion process. It is commonly considered very clean and particle free. However, even bluish flames of natural gas may produce a considerable number of particles with sizes below 10nm, if operating conditions deviate from stoichiometry and mixing at atomic level (D'Anna, 2009).

Pollutants emitted by cooking activities consist of gaseous polycyclic aromatic hydrocarbons (PAHs), nanoparticles and PAHs condensed onto the solid particles (Gao et al., 2015). Their concentration depends on several factors, including ventilation, space confinement, cooking span, air humidity degree and cooking temperature. Emission levels are very low, including nanoparticles which negligibly account for indoor particulate mass. Due to their very low sizes, these nanoparticles however can deposit far inside the airways and potentially reach target organs being dangerous although present in low mass concentration. Many studies demonstrated that there is a correlation between cooking emissions and lung cancer (Gao et al., 2015; Wang et al., 1996; Koo et al., 1996; Kennedy, 2006; Xu et al., 1989). In fact, because of long exposition,

serious respiratory diseases occur in female population and in people working in commercial kitchens (Yu et al., 2015). Also, the stove design, stove temperature, and cooking pot temperature influence the mass and size of particles emitted by biomass cook stoves (L'Orange et al., 2012).

In order to cope with all these variables, this work is focused on the study of particulate generated from gas cooking appliances in two different configurations: cooktop burner with a pot on the fire and a free flame. The exhausts have been characterized in terms of gas-phase composition, including PAHs, and particle size distributions. In addition, particles have been collected in order to make them available for in vitro toxicological studies and to analyse their possible effects on growth and secretion of cytokines, chemokines and growth factors networks production in keratinocyte cells.

2. Experimental

2.1 Experimental system and particle collection

A mid-range cooktop burner fed with network natural gas, which contains methane, and ethane, lower percentages of higher alkanes, has been used. A quantity varying from 20 to 30 ppm of sulfur is present in the natural gas in form of volatile sulfur compounds, specifically mercaptans.

The burner has been used at full power having only the largest burner ignited. The burner was used in two operating conditions: a free flame and a flame with a pot on the fire. Indeed the presence of a relatively cold surface on the flame might quench the combustion reactions and favor the formation of pollutants different from the ones produced in free flame operations or even produce additional ones.

In the "free flame" configuration, a pyrex hood is located at 60 cm from the cooktop; in the "pot on the fire" configuration, combustion-generated materials are sampled just above the pot, at about 30cm. Sampled gases are then flow through an adsorbent resin trap and subsequently passed through an ice trap before exhausted. Sampled exhaust gases are diluted with ambient air during collection by a factor of 2 estimated based on CO₂ measurements.

2.1.1 On-line measurements of particles and detection of contaminant particles

The size distribution of the particles generated during combustion has been measured on-line by a Differential Mobility Particle Sizer (DMPS). The DMPS measures the electrical mobility of an aerosol. It is equipped with a bipolar Am-241 charger and Faraday cup electrometer detector. The DMPS was run with a high carrier gas flow rate (50 l/min) in order to reduce diffusional effects. The size distribution measured by DMPS gives the size of the particles contained in the sampled aerosol, and from the peak area, the concentration of particles in the aerosol. Measurements have been also performed by having the flame switched-off in order to check the possible presence of contaminant particles in the sampled air

2.1.2 Water-based sampling system for particles

In previous studies performed on lab-scale premixed flames in fuel-rich conditions, we have shown that UFPs (Ultrafine Particles) have hydrophilic properties (Sgrò et al. 2012). Consequently, by allowing interaction between nanoparticles and water, e.g., water produced by hydrocarbon oxidation or additional bi-distilled sterilized water added in the ice trap, nanoparticles can be removed from the combustion exhausts and isolated in samples for chemical and morphological characterization. Moreover, the sampling procedure isolates flame-generated nanoparticles from soot and low molecular weight gas-phase organic products.

Water is used as a suspending medium to accumulate and store nanoparticles for long periods of time (days to months) and to investigate their toxicity in toxicological assays. The procedure for collecting combustion-generated particles in water and eliminating volatiles by rotary evaporation or nitrogen purging offers a way of dispersing particles in water suspensions without the use of filters.

Two mechanisms can be considered for trapping particles in water samples. The first one is based on absorption: a gas sample is bubbled through water, which acts as an absorbing liquid for small particles and compounds with high solubility in water. The second mechanism is based on condensation: the gas sample is forced to flow through a cold trap, creating a supersaturated environment, and water condensation on cold walls or on hygroscopic nuclei enables the scavenging and collection of nanoparticles, which remain trapped in the water reservoir. The water sampling enables the capture of nanoparticles, which would require supersonic flow and extremely high pressure drops to be collected/concentrated by impactors and which seem to escape filters.

In order to remove volatile compounds dissolved in water, the samples are partially evaporated under low pressure. During evaporation, performed by continuously reducing pressure from the atmospheric one to few millibar, we observe that the pressure reduces down to about 200-100 mbar, where it remains constant while a boiling stage is observed. The boiling stage is rapid, and the amount of liquid before and after the bubbling remains relatively constant. This boiling stage is attributed to the removal of gaseous species like CO₂ and semivolatile organic species. After the boiling stage, the pressure continues to decrease down to a value of

the order of tens of mbar, where it remains almost constant showing the almost complete removal of the gaseous species dissolved in water.

The samples were subsequently concentrated to the desired concentration value by heating the water sample at a temperature of 75°C, at atmospheric pressure, until the final concentration was reached.

During the concentration procedure, the samples were analyzed every two hours by measuring UV-visible absorption and scattering in order to check the possible transformation of the sample for effect of heating. UV-Visible measurements have shown that the sample was not modified in its chemical nature whereas the scattering measurements have shown that the size of the particles remained unchanged.

We have estimated the collection efficiency of the water-based sampling method by inserting the DMPS downstream of the bubbling reservoir and measuring the size distribution of the particles present in the sampled gases without the condenser/bubbler in the sampling line, with the empty condenser/bubbler in the ice bath, and with the condenser/bubbler filled with 50 ml of water and positioned in the ice bath. The efficiency of particle collection due to the interaction with water can be estimated from the size distribution function of the particles in the gas flow entering the DMPS with and without the water in the reservoir by measuring the reduction in the total number, $\Delta N/N$, or concentration, $\Delta C/C$ of the particles produced by the presence of water. Comparable results in the order of 40% are obtained for both efficiencies so defined.

2.1.3 Chemical and morphological characterization of the sampled particles

The chemical and morphological characterization of the material collected in the form of a water suspension has been performed by using an array of spectroscopic, chromatographic, spectrometric and microscopic analyses. In particular we have used UV-Visible absorption for the chemical characteristics of the collected material, total organic carbon determination (TOC) for the evaluation of the amount of collected carbonaceous material, mass spectrometry (inductively coupled plasma mass spectrometry ICP-MS) and ionic chromatography (IC) for the detection of metal and sulfur and nitrogen components, respectively, and dynamic light scattering (DLS), electro-spray differential mobility analysis (E-DMA) and atomic force microscopy (AFM) for the particle morphology.

2.1.4 Chemical characterization of light aromatic compounds

Light organic compounds have been sampled by adsorbing them on a XAD2 resin. The resin was dissolved in a solution of 0.5 ml of dichloromethane. Species identification was made by gas chromatography. HP5890 gas chromatograph equipped with an HP-5MS crosslinked 5% PhMe siloxane 30m x 0.25mm x 0.25mm film thickness column coupled with an HP5975 mass spectrometer with an electron impact/chemical ionization ion source has been used. Species identification was made by comparison with mass spectrum in Wiley computer library with 138.000 spectra. PAHs have been quantified individually by using the factors response of standard mixtures of twenty-five PAHs.

Quantification and characterization of the species collected in the water samples have been performed by UV-Vis absorption, inductively coupled plasma mass spectrometry (ICP-MS) analysis and ionic chromatography (IC) analysis.

2.1.5 In vitro toxicity testing

The immortalized human keratinocyte cell line (HaCaT) has been used for toxicology tests. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) including 2 mM L-glutamine (Lonza) that was supplemented with 100 U/mL penicillin (Lonza) and 100 mg/mL of streptomycin (Lonza) and 10% heat-inactivated fetal bovine serum (Gibco) in a humidified atmosphere (95% air/ 5% CO₂) at 37°C. Culture media and supplements were purchased from Life Technologies (Paisley, UK), and fetal calf serum (FCS) was from Hyclone Lab (Logan, UT). Cells were seeded at a density of 10⁵ cells in 75 cm² flasks to grow to confluence. The medium was changed twice a week.

2.1.6 Cell viability assays

For viability/growth inhibition assay, HaCaT cells were seeded in 96 well plate at a density of 3x10⁴ cells/well. After 24 h, culture media were replaced with media containing nanoparticles in "free flame" condition at concentrations of 0.075, 0.15, 0.3 ppm and in the "pot on the fire" condition at concentrations of 0.1, 0.2 and 0.4 ppm, corresponding to 20, 40 and 80 µL of stock solution of nanoparticles. Cells were exposed to nanoparticles for 24 h. Cell viability was evaluated with crystal violet, which correlates optical density with cell number, according to the procedure described by Kueng et al. (1989). In detail, cells were washed with PBS 1X and fixed by adding 50 µL of a 10% formalin solution. After 15 min cells were washed with deionized water and stained with 50 µL of 0.1% crystal violet solution in water for 30 min. Excess dye was removed by washing with deionized water and plates were air-dried prior to bound dye solubilization in 50 µL of 10% acetic acid. The optical density of dye extracts was measured at 595 nm using a microplate reader (DAS, Italy). All experiments were performed in triplicate and repeated at least 3 times.

2.1.7 Bioplex cytokine/chemokine detection

In order to investigate the inflammatory effects, HaCaT cells were seeded in 6 well plate at a density of 2×10^5 cells/well in DMEM 10% FCS. After 24 h, cells were washed twice with PBS and culture media were replaced with media containing nanoparticles at concentration of 0.1 ppm in DMEM 0% FCS, in order to avoid bovine serum interference with Bioplex assay. Cells were exposed to nanoparticles for 24 h.

After 24 h exposure, 1 mL medium was sampled and immediately frozen and stored at -70°C until the assay was performed. The detection of pro- and anti-inflammatory cytokines released into the culture medium was carried out with the Bio-Plex Pro Human Cytokine 27-Plex Panel for the detection of interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, Eotaxin, Granulocyte-colony stimulating factor (G-CSF), Granulocyte macrophage colony stimulating factor (GM-CSF), Interferon (IFN)- γ , IFN- γ inducible protein 10 (IP-10), Monocyte chemo attractant protein 1 (MCP-1), Macrophage inflammatory protein 1-alpha/beta (MIP-1 α , MIP-1 β), RANTES, Tumor necrosis factor alpha (TNF- α), Platelet-derived growth factor (PDGF-bb), Vascular endothelial growth factor (VEGF) and basic Fibroblast growth factor (bFGF) (BioRad).

Data were acquired using a Bio-Plex 200 system equipped with Bio-Plex Manager software v5.0 (BioRad). All washing steps were performed on the Bio-Plex magnetic wash station (BioRad). Measurements were performed on single spent medium sample diluted (1:2) using the Bio-Plex 27-plex human cytokine kit from BioRad according to the manufacturer's protocol. A sample of DMEM alone was also run to account for background levels of secreted proteins. Data are expressed as pg of cytokine/mL of conditioned medium (mean \pm SEM), with N= 3. The standard curves optimization and the calculation of analyte concentrations were performed by using the Bio-Plex Manager software.

3. Results and discussion

Concentrations of PAHs were measured at the exhausts of the cook-top burner with a without the pot. Although present in very low mass concentrations, 5 ng/l and 3.2 ng/l with and without the pot on the burner, respectively, PAHs are emitted during indoor activities. Figure 1 reports the molar fractions of low and high molecular weight PAHs. The distribution of PAHs shows a prevalence of naphthalene in the PAH inventory and the slightly prevalence of high-molecular weight PAHs in the "pot on the fire" condition.

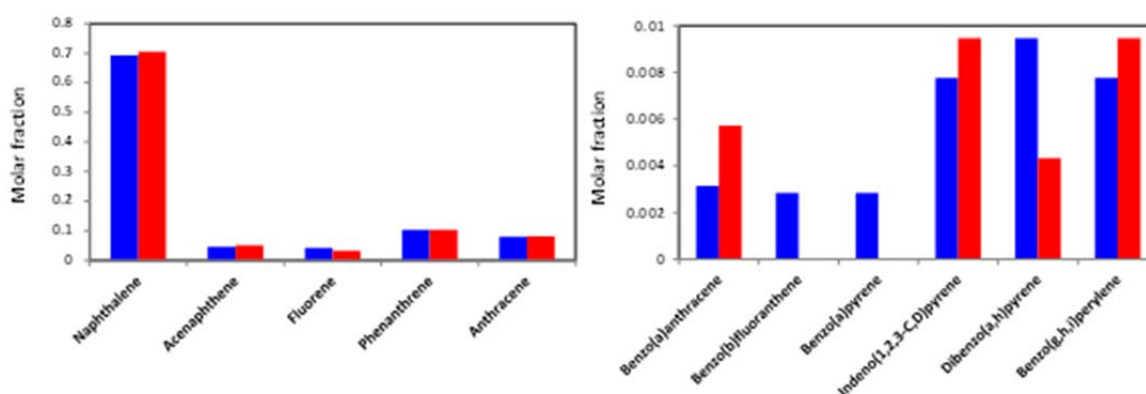


Figure 1. Molar fraction of low (left panel) and high (right panel) molecular weight PAHs (red "free flame"; blue "pot on the fire").

Particles generated during combustion has been measured on-line; figure 2 shows the results of the measurements. Data show that particles with sizes below 1nm are due to instrument artefacts (ion-clusters). Clearly, the flame produces and emits particles larger than 2.5 nm and below 10 nm. Their mass concentration is of the order of few ppb (1.5ppb), but they are present in huge number concentration (5×10^{13} #/cm³).

Although particles are emitted in very low concentrations, their effect on the indoor ambient is appreciable. We have measured the size distribution function of the particulate matter in our lab with fresh air at about 5 meters from the cooktop (the flame was switched-off for about 2 days and the windows remained open for two days so that the ambient air was completely changed). Thereafter we have switched-on the flame on the cooktop burner and measured the particle size distribution after 1 day of operation and after 3 days. Figure 2 reports also the particle size distribution functions measured in clean air in the lab showing that in clean ambient air the particle number is below the detection limit of the DMPS whereas particle in the size range typical of those measured at the exhaust of the cooktop burner are accumulated in the lab after 1 and 3 days of operation.

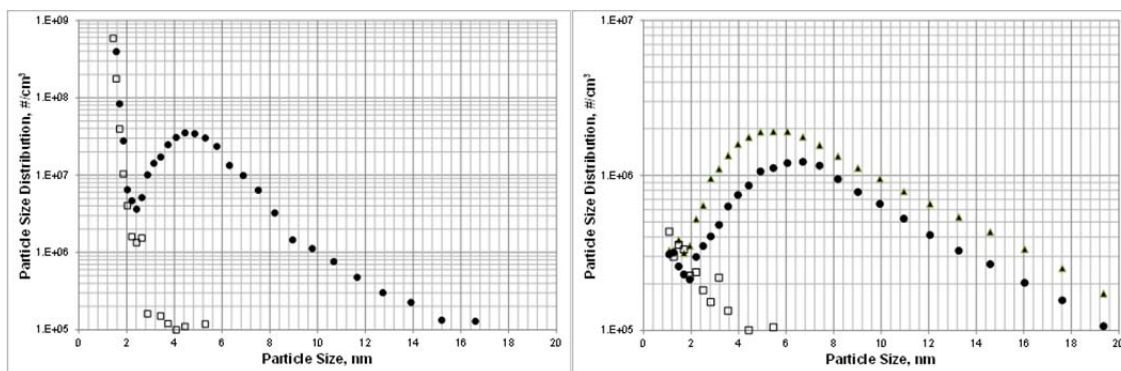


Figure 2. Size distribution functions of the particles in the exhaust gases. Left pane: with the flame switched-on (full circles) and the flame switched-off (full squares). Right panel: in air after 1 (circles) and 2 (triangles) days of flame operation.

The chemical characterization of the collected material have been performed by UV-vis absorption, ICP-MS and IC. Results have shown that carbon nanoparticles of organic carbon and nitrates and sulfates are present in the collected material.

The spectroscopic characteristics of the sampled material suspended in water, determined by UV-visible light absorption technique, are somewhat similar to those measured “in situ” in non-sooting laminar premixed hydrocarbon flames (Sgrò et al., 2012). In particular, UV absorption spectrum of water-sampled material has a maximum around 200 nm and decreases very fast in the near UV. It is completely different from the typical absorption spectrum due to soot, which exhibits a broad maximum at 250 nm and decreases toward the visible with an inverse power law. The intensity of the 200nm-band does not decrease when the water-sample is subjected to the degassing process by means of a rotary evaporator indicating that this absorption band is not due to gaseous compounds dissolved in the water sample.

The strong absorption bands centred at about 200 nm measured in the sample in water suspensions is typical of nitrogen-containing compounds, such as nitric acid, deriving from NO_x interaction with water. This peak is superimposed to a continuous background of absorption very similar to the light absorption spectrum of nanoparticles of organic carbon, i.e., clusters of high-molecular mass aromatic molecules held together by van der Waals interactions, measured in rich flames. From these absorption spectra, the nanoparticle concentrations have been estimated taking into account the optical properties, i.e., the absorption coefficient in the UV, of combustion generated nanoparticles.

ICP-MS measurements performed on the water samples have shown that metals are present in trace amount and cannot be the source of nanoparticles detected in the water samples.

To evaluate the presence of nitrates and sulphates, an ionic chromatography (IC) analysis has been performed. The chromatograms clearly show the presence of noticeable amounts of nitrates and sulfates deriving from the interaction of flame generated NO_x and SO_x with water. Their concentrations are however of the same order of magnitude of those found in drinkable water.

Regarding biological effects, results of crystal violet assay obtained on the material collected in “free flame” and in “pot on the fire” conditions have showed shows no relevant reduction in cell number after 24h of treatment. It is interesting to note a little positive effect in increasing cell number (+20%) at the lowest concentration, as shown in Fig. 3.

Moreover, secretome analysis showed that of the 27 cytokine tested was possible to dose 21 cytokines, released into the culture medium after 24h of exposure to 0.1ppm of nanoparticles (corresponding to 20 μL of stock solution of nanoparticles). No relevant overexpression or downregulation is noted on the secretion of these cytokines (data not shown).

These results are in contradiction with biologic effects observed by Pedata et al. (2013) on the same cell lines when treated with nanoparticles formed in bluish lab flames suggesting that nanoparticles formed in gas cooking devices have peculiar characteristics. It is important to study the change of nanoparticle characteristics when the flame structure changes for effect of putting a pot and even food on the cook stove, i.e., in operating conditions closer to the real ones

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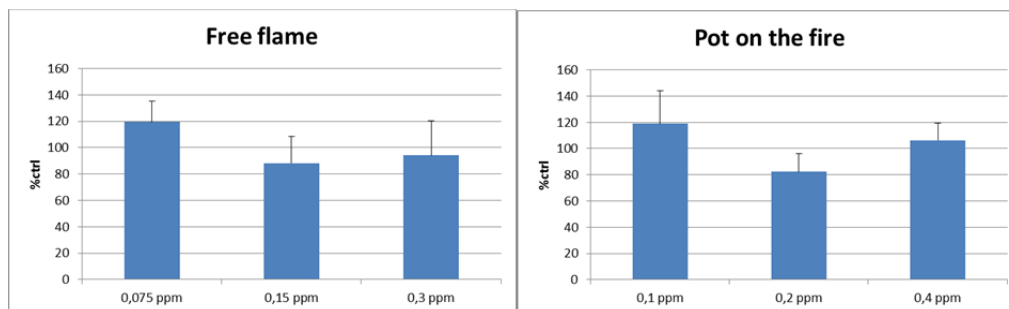


Figure 3. Cell viability assay on HaCaT cells, 24 h of treatment

4. Conclusion

Measurements show that the flame produces and emits particles in the size range 2.5-20 nm. Their mass concentration is of the order of few ppb, but they are present in huge number concentration.

The chemical and morphological characterization of the collected material has shown that carbon nanoparticles of organic carbon - 3 to 20 nm- and nitrates and sulfates are collected.

Regarding biological effects, results indicate that both material collected in “free flame” and “pot on the fire” have not effects on cell viability of HaCaT cells; moreover, results of secretome analysis suggest that the pro-inflammatory pathway is not activated.

Further studies are in progress on A 549 cells (human alveolar epithelial-like cells) to evaluate the other important route of exposure to sub-10nm particles.

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