# Soot-driven reactive oxygen species formation from incense burning

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ABSTRACT

This study investigated the effects of reactive oxygen species (ROS) generated as a function of the physicochemistry of incense particulate matter (IPM), diesel exhaust particles (DEP) and carbon black (CB). Microscopical and elemental analyses were used to determine particle morphology and inorganic compounds. ROS was determined using the reactive dye, Dichlorodihydrofluorescin (DCFH), and the Plasmid Scission Assay (PSA), which determine DNA damage. Two common types of soot were observed within IPM, including nano-soot and micro-soot, whereas DEP and CB mainly consisted of nano-soot. These PM were capable of causing oxidative stress in a dose-dependent manner, especially IPM and DEP. A dose of IPM (36.6–102.3 µg/ml) was capable of causing 50% oxidative DNA damage. ROS formation was positively correlated to smaller nano-soot aggregates and bulk metallic compounds, particularly Cu. These observations have important implications for respiratory health given that inflammation has been recognised as an important factor in the development of lung injury/diseases by oxidative stress. This study supports the view that ROS formation by combustion-derived PM is related to PM physicochemistry, and also provides new data for IPM.

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## 1. Introduction

A growing body of epidemiological and clinical evidence has led to increased concerns regarding the potential harmful effects of PM on health. Particulate pollutants are associated with increased risk of pulmonary and cardiovascular hospital admissions, morbidity and mortality, especially when PM with an aerodynamic diameter less than 2.5 µm (PM$_{2.5}$) originates from combustion sources (Dominici et al., 2006).

Particulate-induced health effects are considered to be driven by the production of ROS in respiratory environments, causing inflammatory reaction and concomitant injury and disease (Poli and Parola, 1997). The formation of hydroxyl radicals (•OH) is considered pivotal to the oxidative capacity of PM. Hydroxyl radicals are able to attack deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Yu and Anderson, 1997), and consequently can cause oxidative nucleotide alteration leading to cancer (Vallyathan et al., 1998).

PM$_{2.5}$ is capable of generating large quantities of radicals (Dellinger et al., 2001). Such radicals can result in oxidative stress in the lung environment depending on particulate physicochemical properties, such as particle size, surface area (SA) and composition (Donaldson et al., 1996). Transition metals contained within PM, for example, are available for redox-cycling (Gurgueira et al., 2002), which can generate superoxide and hydroxyl radicals (Donaldson

et al., 1997). ROS generation may also be correlated to PM type and morphology. In particular, soot has been identified as a platform for ROS generation (Rouse et al., 2008).

For many centuries, incense has been burnt for religious ceremonies, therapy and to perfume the air; it emits a significant fine-sized particulate pollutant (Lin et al., 2008). When incense is burnt, the resultant smoke contains a mixture of PM, gases and organic compounds; these have previously been linked to irritant respiratory effects (Ho et al., 2005) and cancer development (Friborg et al., 2008). These health effects may be related to ROS formation. Research has shown that smoke from incense burning was able to raise significant 8-Oxo-2'-deoxyguanosine levels (a ROS biomarker) and DNA strand breaks in temple workers (Navasumrit et al., 2008). However, our current knowledge of particulate oxidative capacity is mainly based on non-incense studies, using surrogate observations from PM studies on indoor and outdoor air (BéruBé et al., 2004).

To understand how PM impacts on human health, the physicochemical properties must be firstly characterised. Combustion-derived particles from different sources vary considerably with respect to size distributions, morphologies and elemental composition. Some particle types have been extensively-researched and have well-understood morphologies; for example, DEP is known to consist of nano-sized, spherical, carbon particles observed as individual particles, small and large chains or agglomerates (BéruBé et al., 1999) with metals and chemicals on its surface (Aam and Fonnum, 2007). CB is an industrial carbon produced by the thermal decomposition of hydrocarbons and is composed of spherical nano-soot in different shaped agglomerates (Zhu et al., 2004), typically with chloride and

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sulphur on its surface (Zielinski et al., 1999). Our comparison control particles were DEP (positive control), which has been identified as a common air pollutant associated to ROS formation, and CB, a commonly used negative control particle for toxicological investigations of PM bioreactivity (Bérubé et al., 2007). IPM contains metals and organics due to the diverse range of composite materials (e.g. wood and resins) used to make incense (Lin et al., 2008). This study aims to investigate the oxidative capacity of IPM as a function of its physicochemistry; with comparisons made with DEP and CB.

In order to achieve the objectives of this study, transmission electron microscopy (TEM), coupled with energy dispersive X-ray (EDX), was used to identify particle types; field emission scanning electron microscopy (FESEM) and image analysis were utilised for morphological measurement; inductively coupled plasma mass spectrometry (ICP-MS) was employed to determine metals. The DCFH assay and PSA were selected to determine ROS formation.

## 2. Experimental section

### 2.1. Particle collection and preparation

Three different types of joss sticks, purchased in a Taiwanese incense shop, were used in this study (Table S1). Two major fragrant materials were used in the incense, sandalwood (Type A and C) and agarwood (Type B); all of them were the same length, including the combustible section. It was noted that whereas Type A and B weighed the same; Type C was notably heavier. All the burning processes of incense ($n = 4$; three joss sticks for each collection) were performed in a polyethylene terephthalate tube ($0.0032$ m$^3$; length: $0.41$ m, diameter: $0.1$ m) at an average temperature of $25$ °C and $55\%$ relative humidity, whilst at a constant burning time (and therefore rate), inside a dedicated fume cupboard located in a laboratory at the School of Biosciences (Cardiff University). The emitted smoke was directly introduced into the sampling systems for 30 minute collections using an enclosed system with filtered air (Chuang et al., In Press). To avoid cross-contamination, the ventilation was turned on 30 min before and left to run 30 min after incense burning. DEP was collected 10 cm from the exhaust system and directly into the emissions stream (Bérubé et al., 1999) from the exhaust fumes generated by a diesel van (CDI, Mercedes-Benz Sprinter) with the engine idling. Low-sulphur diesel was used for the van fuel. The emitted fumes were directed to the samplers. The chances of any measureable background contamination using this system was effectively nil.

Two different air sampling systems were used for this study. A 30 l/min air pump (JD Technical, UK) attached to a PM$_{10}$ selective-inlet head (horizontal elutriator; C30 Classifier; Thermo, UK) was used to collect PM on 47 mm diameter polycarbonate filters ($0.67$ μm pores; Millipore, UK) for the purpose of physical and chemical descriptions (Bérubé et al., 1999). For the determination of particle oxidative capacity, PM$_{10}$ were collected using an Airborne Sample Analysis Platform system (ASAP; Model 2800 Thermo, USA) on polyurethane foam substrates (PUF; Rupprecht & Patashnick Co. Inc., USA) with a high sample flow-rate of 200 l/min. A near pure manufactured CB (Monarch 120; Cabot Corporation, UK) was selected as a control particle (Murphy et al., 1998). PM for the purposes of characterisation and oxidative potential were sampled in parallel from the same burning experiment.

### 2.2. TEM and elemental analysis

The prepared TEM grids were analysed using a Philips CM12 TEM at an accelerating voltage of 80 kV, spot size 1. The images were recorded on a SIS MegaView III Digital Camera. X-ray Microanalysis (XRMA) was performed using an EDX analysis Genesis System.

### 2.3. FESEM and image analysis

The samples were carbon coated to an average thickness of 10 nm prior to analysis using a sputter coater (Biorad, UK). The microscope used was a FESEM (Philips XL30; Philips Electron Optics, NL) at an accelerating voltage of 25 kV, spot size 3. This study did not measure physical parameters of CB (M120) due to this particle having been well-described in previous studies (Murphy et al., 1998; Zhu et al., 2004).

### 2.4. ICP-MS

The elements (Mg, Al, P, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Mo, Cd, Sn, Ba, Hg and Pb) were determined by ICP-MS (PerkinElmer Elan 500). The samples were digested with concentrated nitric acid (Fisher Primar grade, specific gravity 1.48) carried out in a CEM MDS-200 microwave system, using CEM advanced Telfon-lined composite vessels (Jones et al., 2006). The pressure was increased to 80 pounds per square inch (psi) for approximately 20 min, producing a digestion temperature of approximately 180 °C. Samples were diluted to 10% nitric acid using deionised ($>18$ MΩ) water. Nitric acid blanks were run to detect any contamination during the analysis process. A solution of a certified rock standard (BCR1) was used to check the accuracy of the analyses.

### 2.5. DCFH assay

ROS was determined using a cell-free DCFH assay (Koshy et al., 2007). Dichlorodihydrofluorescin (DCFH) was prepared by 2′,7′-dichlorodohydrofluorescin diacetate (DCFH-DA; Sigma-Aldrich, UK), since this compound has been widely used as an indicator of reactive species formation (Hung and Wang, 2001). 2 ml of 0.01 N sodium hydroxide (NaOH) was added to 0.5 ml of 1 mM DCFH-DA in methanol. After standing at room temperature in the dark for 30 min, 10 ml of 25 mM sodium phosphate monobasic (NaH$_2$PO$_4$), pH 7.4 was added to provide a 40 μM stock solution of activated DCFH. The reagent was added to 5, 15, 50 and 150 μg/ml of different sources of PM ($n = 9$) suspensions in Tris–HCl buffer (pH 7.4) and incubated at 37 °C for 25 min in light-resistant Eppendorf tubes.

The fluorescence intensity was measured with a Cary Eclipse Fluorimeter (Varian Instruments, CA, USA), at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. In order to relate the fluorescence intensity with the hydrogen peroxide (H$_2$O$_2$) concentration, a linear calibration curve of H$_2$O$_2$ was performed with 40 mM Tris–HCl containing 1 μM activated DCFH solution (See et al., 2007). The calibration concentrations were spiked with 30% H$_2$O$_2$ ranging from 0.1 to 300 μM prior to the addition of 10 μM FeSO$_4$ to give a final volume of 1.5 ml. All DCFH assay data was presented as oxidative capacity equivalent to H$_2$O$_2$ concentration for each particle type, after being corrected for blanks (See et al., 2007).

### 2.6. PSA

The PSA is a method that uses a biological indicator to assess the percentage oxidative DNA damage caused by PM. The plasmid ΦX174 RF DNA molecule (Promega, UK) has previously been shown to be susceptible to damage by ROS and metals (Shao et al., 2006). PM samples were prepared from a stock particle at different concentrations (5–1000 μg/ml) in molecular grade water (Sigma-Aldrich, UK). 20 ng of ΦX174 RF DNA was added in the final volume and incubated with PM ($n = 9$). A negative control was established using incubations of DNA in molecular grade water and the positive controls were incubated with the restriction enzyme Pst I (Promega, London, UK). Before loading 3.33 μl dye (Promega, UK), all samples were gently agitated for 6 h (Koshy et al., 2007). Samples were electrophoresed on a gel (0.6% Agarose; Bioline, UK) with 0.25% Ethidium bromide