SCIENTIFIC PAPER

APPLICATION OF PROFLUORESCENT NITROXIDES FOR MEASUREMENTS OF OXIDATIVE CAPACITY OF COMBUSTION GENERATED PARTICLES

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Received 13.01.2012

Revised 26.06.2012.

Accepted 20.08.2012.

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Part of this paper was presented at the Scientific Meeting Particulate Matter: Research and Management, 3rd WeBIOPATR Workshop & Conference, Belgrade, Serbia , 15-17 November 2011

ABSTRACT

Oxidative stress caused by generation of free radicals and related reactive oxygen species (ROS) at the sites of deposition has been proposed as a mechanism for many of the adverse health outcomes associated with exposure to particulate matter (PM). Recently, a new profluorescent nitroxide molecular probe (BPEAnit) developed at QUT was applied in an entirely novel, rapid and non-cell based assay for assessing the oxidative potential of particles (i.e. potential of particles to induce oxidative stress). The technique was applied on particles produced by several combustion sources, namely cigarette smoke, diesel exhaust and wood smoke. One of the main findings from the initial studies undertaken at QUT was that the oxidative potential per PM mass significantly varies for different combustion sources as well as the type of fuel used and combustion conditions. However, possibly the most important finding from our studies was that there was a strong correlation between the organic fraction of particles and the oxidative potential measured by the PFN assay, which clearly highlights the importance of organic species in particle-induced toxicity.

KEY WORDS: combustion particles, diesel particles (DPM), oxidative stress, reactive oxygen species (ROS).

1. INTRODUCTION

Particulate pollution has been widely recognised as an important risk factor to human health with $3.7 billion spent on respiratory diseases in Australia alone. Epidemiological studies have established strong associations between exposure to ambient particulate matter and increased respiratory and cardiovascular disease morbidity and mortality, particularly among individuals with pre-existing cardiopulmonary diseases [1]. Recently the International Agency for Research on Cancer (IARC), which is part of the World Health Organization (WHO), classified diesel engine exhaust as carcinogenic to humans (Group 1) on the 12th June 2012, based on sufficient evidence that exposure increases risk for lung cancer. To develop methods that could help to mitigate the adverse health outcomes induced by PM, it is important to know the PM properties and the mechanism(s) that are responsible for PM toxicity. Identification of the PM properties that are the most relevant for promoting adverse health effects is crucial not only for our mechanistic understanding but also for the implementation of strategies for improving air quality. Despite the availability of a huge body of research, the underlying toxicological mechanisms by which particles induce adverse health effects are not yet entirely understood.

One of the important aspects of environmental sciences in the last decade was to identify the physical and chemical characteristics of ambient PM responsible for its health effects and within that scope, particle size, surface area and chemical components, such as metals and certain classes of organics (e.g. quinones) have been implicated in PM-induced health effects and more specifically, in the generation of reactive oxygen species (ROS).

ROS can be formed endogenously, by the lung tissue cells, during the phagocytic processes initiated by the presence of PM in the lungs, or by particle-related chemical species that have the potential to generate ROS. In addition to the particle-induced generation of ROS, several recent studies have shown that particles may also contain ROS (so called, exogenous ROS). As such, they present a direct cause of oxidative stress and related adverse health effects and the hypothesis that particles contain or produce ROS is the driving force for this research project.

It is a reasonable assumption that exogenous ROS can cause the same responses (oxidative stress) in the cell as endogenously formed ROS. Therefore, a rapid screening assay able to

evaluate PM oxidative potential in terms of their inherent ROS, and therefore their ability to cause oxidative stress, would be beneficial for gaining better understanding about the nature of the particles most relevant for their negative health impact. Such a screen would also provide a helpful tool in efforts to further improve air quality and protect public health. To address this need we have developed a methodology for quantitative detection of the oxidative capacity of airborne nanoparticles based on in-house developed profluorescent nitroxide molecules. This methodology has been evaluated on combustion-generated particles. Correlations between various particle properties and their oxidative capacity, as measured by our molecular probes, will be discussed.

2. METHODOLOGY

Cellular responses to oxidative stress have been widely investigated using various cell exposure assays ([2-5]. However, in order to provide a rapid screening test for the oxidative potential of PM, less time-consuming and cheaper, cell-free (or acellular) assays are necessary.

The only analytical approach that permits the direct detection and quantification of radical species is electron paramagnetic resonance (EPR). This method allows the quantification as well as specific identification of the free radical species generated when specific spin traps or probes are used in the combination with specific reagents. Apart from the complexity and high price of the instrument, a potential pitfall of EPR-based measurements of ROS formation by nanoparticles may result from chemical or physical interference with spin-trapping agents, and could be checked by the analysis of specific ROS donor systems (e.g. xanthine /xanthine oxidase, H_2O_2/Fe) spiked with nanoparticles [6]. Several cell-free approaches have been used to explore oxidative potential of PM in a quantitative manner. They all have certain limitations, do not provide directly comparable results and, to date, none of these assays has been acknowledged as the best acellular assay and none have yet been widely adopted for investigation of potential PM toxicity.

A number of assays are available such as DTT[7], POHPAA[8], DCFH[9], DHR-6G[10] assays as well as the ascorbate depletion test[11].

However, DTT is reactive towards limited number of species, it requires an additional step that may be a potential source of an experimental error and also the usage of this probe requires an incubation time of up to 90 mins [7]. On the other hand, DCFH is prone to autooxidation and thus brings into question the suitability of this assay. Also, DHR-6G is air

sensitive and photo-sensitive which limits its performance as either oxygen or light can produce significant background fluorescence

Out of all of the assays the fluorescence-based ones have been most commonly used in the quantification of PM-related ROS, primarily due to the high sensitivity of fluorescence detection. They are based on non-fluorescent or weakly fluorescent molecules that yield fluorescent products upon reacting with ROS.

Nitroxides are well-known as effective quenchers of excited states of fluorescent moieties. During the past seven years numerous nitroxides have been synthesized at QUT [12]. These nitroxides possess fluorophore covalently bound within the structure whereas most of the other nitroxide-fluorophore adducts used by other researchers have labile covalent linkages that are prone to hydrolysis and resulting separation of the nitroxide from the fluorophore. This important feature makes the QUT probes superior to previously synthesized nitroxides because of their enhanced chemical and thermal stability. All these nitroxide containing fluorophores display substantial fluorescence suppression. Also, they have the same excitation and emission maxima as the fluorophore itself. Some of these nitroxides, synthesized at QUT are shown in the Figure 1 together with their excitation and emission wavelengths. These molecules react with radicals, leading either to reduction of the nitroxides to the hydroxylamines or oxidation to oxoammonium cation. The measure of the number of radicals trapped by the nitroxides or other redox reactions that occur is the intensity of the fluorescence emission. These nitroxides are classified as profluorescent according to the fact that they are initially weakly fluorescent, but can be transformed into a fluorescent form after a simple chemical reaction. Taking into account the above, these molecules can serve as powerful optical sensors applicable as detectors of free radicals and dynamic fluorescent indicators of the overall redox environment in cellular systems (redox active agents).

Figure 1.

A number of profluorescent nitroxide probes were evaluated [13] for their ability to detect and quantify ROS associated with combustion generated particles. Out of all of the evaluated probes 9,10-bis(phenylethynyl)anthracene-nitroxide (BPEAnit) was chosen as the most appropriate for use with combustion generated particles [14]. The excitation and emission

wavelength of the BPEAnit are long enough to avoid overlapping with the background fluorescence coming from optically active compounds which may be present in PM.

BPEAnit has been applied *in situ* to assess the oxidative potential of cigarette smoke [14], diesel particle matter (DPM) [15-17] and wood smoke [18]. Samples were collected by bubbling aerosol through an impinger containing 20 mL of 4 μM BPEAnit solution (using AR grade dimethylsulphoxide as a solvent) followed by fluorescence measurements with a spectrofluorometer (Ocean Optics). The amount of BPEAnit reacting with the combustion aerosol was calculated from a standard curve obtained by plotting known concentrations of the methyl adduct of BPEAnit (BPEAnit-Me; fluorescent) against the fluorescence intensity at 485 nm. For each setting and particulate source, two samples were taken. The first one was the result from the exposure of BPEA solution to the particle-free gas phase, which was done by placing HEPA-filter between an impinger and an aerosol source. Test sample was collected upon exposure to both the particle and the gas phase, demonstrating the effect of the particle-related ROS. Based on the difference in fluorescence signals at 485 nm between the test and HEPA-filtered control sample, the amount of particle-associated ROS emitted for each test sample was calculated and normalised to the particle mass to give ROS concentrations (nmol/mg).

3. RESULTS AND DISCUSSION

To investigate the use of the profluorescent nitroxide BPEAnit to detect ROS present in combustion-generated particles using fluorescence spectroscopy initial experiments were conducted with cigarette smoke. As one of the most common combustion-generated aerosols and due to its easy generation, it was taken as a model aerosol. Sampling mainstream cigarette smoke gave a linear increase of fluorescence intensity with increasing number of puffs with this pattern being reproducible, although values varied with each individual cigarette. Sampling much lower concentrations of particles as produced by sidestream cigarette smoke generated in a test chamber also gave increased fluorescence intensity with increased sampling time. Since the increase of signal was well above the detection limit, we have clearly shown the capability of this approach to be successful in determining the levels and potential toxicological impact of ROS in general studies where near ambient concentrations of particles are observed. By being able to omit the derivatisation step, and by

undertaking fluorescence measurements immediately after the sampling, we demonstrated the potential for these probes for the future development of real time ROS detectors.

The BPEAnit was used to further study the potential toxicological impact of particles produced during biomass combustion by an automatic pellet boiler and a traditional logwood stove under various combustion conditions [18]. The fluorescence of BPEAnit was measured for particles produced during various combustion phases, at the beginning of burning (cold start), stable combustion after refilling with the fuel (warm start) and poor burning conditions. For particles produced by the logwood stove under cold-start conditions significantly higher amounts of reactive species per unit of particulate mass were observed compared to emissions produced during a warm start. In addition, sampling of logwood burning emissions after removing all the semivolatile species resulted in an 80-100% reduction of the fluorescence signal of BPEAnit probe, indicating that the majority of reactive species were semivolatile. A significant reduction in PM oxidative potential after thermal conditioning was also observed by Biswas and co-workers [19] who used a dithiothreitol (DTT) assay to measure the oxidative potential of particulate matter produced by heavy–duty vehicles. As a further support of the role of organic species in particle induced oxidative stress, we observed strong correlations ($r = 0.85$ and 0.99) between the amount of ROS and the mass fraction of organic species in the PM during cold-start stable combustion and warm-start combustion (Figure 2).

Figure 2.

The profluorescent nitroxide probe was also applied to study the oxidative potential of DPM. Emissions from various alternative fuels and diesel engine technologies were investigated. Fuels investigated included ethanol [20], Fischer-Tropsch diesel (gas to liquid) [16] and various biodiesel stocks (soy, canola, tallow) in various blend percentages [15]. A similar picture as with the wood combustion also emerged with a good correlation between the particle volatile organic content and ROS concentration being observed.

Particles from sidestream cigarette smoke were shown to have 4-9 and 30-80 times less ROS per unit of mass than particles produced during warm- start and cold-start logwood combustion, respectively. This finding sheds a new light on logwood smoke particles and draws attention to the importance of expanding the knowledge on the toxicological properties

of wood smoke particles. Diesel exhaust particles generated under full engine load were found to have similar ROS concentrations as sidestream cigarette smoke particle

Figure 3.

These studies also provided an opportunity to look into the correlation between the physical properties of DPM and oxidative capacity of particles measured as the concentration of ROS. Toxicological studies, such as [21], have pointed to the particle surface area as a potential metric for assessing the health effects of PM. The surface area of a particle provides a measure of the ability of toxic compounds (such as PAHs or ROS) to adsorb or condense upon it. Polycyclic Hydrocarbons (PAHs) are the principal pollutants from incomplete combustion, and are of special interest due to their toxicity, carcinogenicity, and ubiquitous presence in the environment [22]. Therefore, a particle's surface area can be viewed as a "transport vector" for many compounds deleterious to human health and requires more detailed analysis.

In addition, it is of urging interest to introduce an effective automated real-time particle-bound ROS sampling system that will allow routine evaluation of health effects and monitoring of the pollution. Following this, improvement in the sampling methodology coupled with the usage of a very sensitive probe such as BPEA nitroxide can provide good ROS monitor. As previously used technique, liquid impingement, has relatively low and size-dependent collection efficiency for particles smaller than 500 nm, we are implementing the usage of particle into liquid sampler (PILS) to overcome this drawback. PILS grows submicron particles in a condensation growth chamber and subsequently collects them using a wetted cyclone [23]. BPEA nitroxide is used to collect particles. This approach makes ROS measurements more efficient, less time consuming and less labor intensive and it is currently being tested.

4. CONCLUSIONS

An in-house developed methodology for detection of PM–derived ROS by using a profluorescent nitroxide probe (BPEAnit) has been developed and provided a good basis for employing the new probe for the assessment of the oxidative potential arising from particles generated by other combustion sources. Considering that for all three aerosol sources (i.e. cigarette smoke, diesel exhaust and wood smoke) the same assay was applied a direct

comparison of the oxidative potential measured for all three sources of particles is possible. s. What is even more important is that a good correlation was observed between the semivolatile organic content of combustion particles (both for wood burning and DPM) and their oxidative capacity as measured through the ROS concentration. This highlights the importance of semivolatiles in the oxidative potential of the particulate matter. This has far reaching consequences on how we regulate particle emissions from combustion sources such as diesel vehicles. For example, the new standards for diesel vehicle engine emissions (EURO 5/6) are based on measurements of particle number emissions and not particle mass emissions. The introduction of particle number based standards as opposed to mass based standards were introduced as the number much better reflects the nanoparticle component of DPM than simple mass based measurements. To achieve reproducible particle number measurements, the standards introduce thermal conditioning of the exhaust prior to sampling. This results in the removal of any semi-volatile organic components from the exhaust particles. If the semi-volatile organic component is responsible for the oxidative capacity of particles, and therefore drives their toxicity, the validity of the new diesel vehicle emission standards has to be brought into question.

5. ACKNOWLEDGEMENT

Parts of this paper was presented at the 3rd WeBIOPATR workshop, Belgrade 15.-17. November 2011. This work was supported by the Australian Research Council Centre of Excellence for Free Radical Chemistry and Biotechnology (CE 0561607), the Australian Research Council Discovery grant (DP120100126) and Queensland University of Technology.

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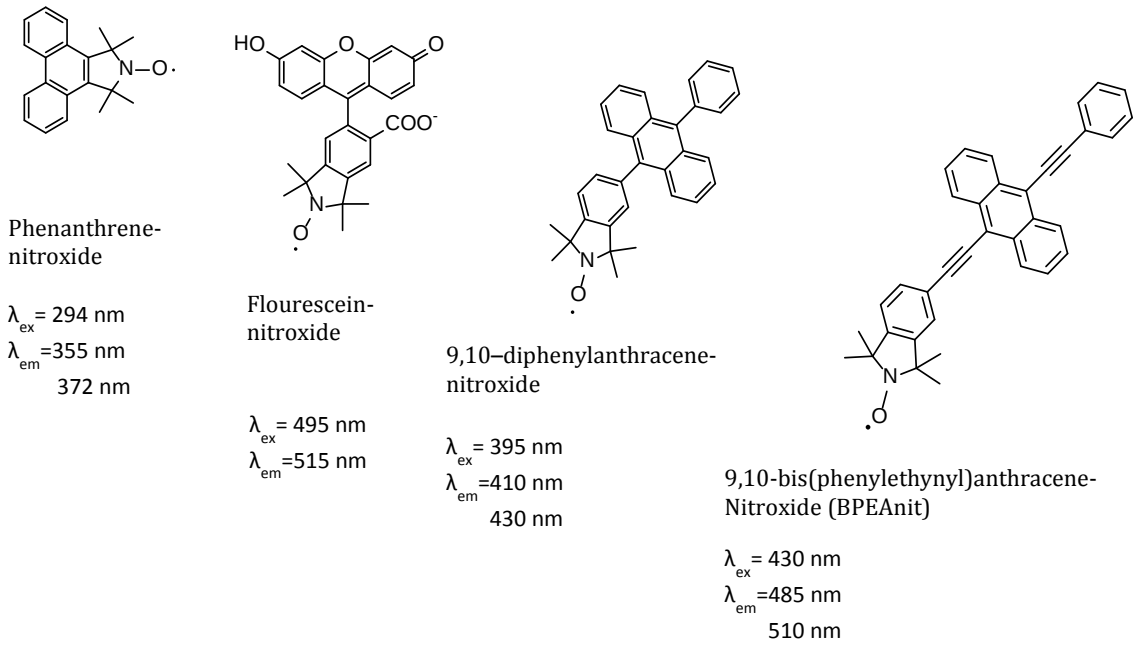

Figure **1** Structures of some of the profluorescent nitroxides synthesised at QUT together with the excitation and emission wavelengths of the fluorophores.

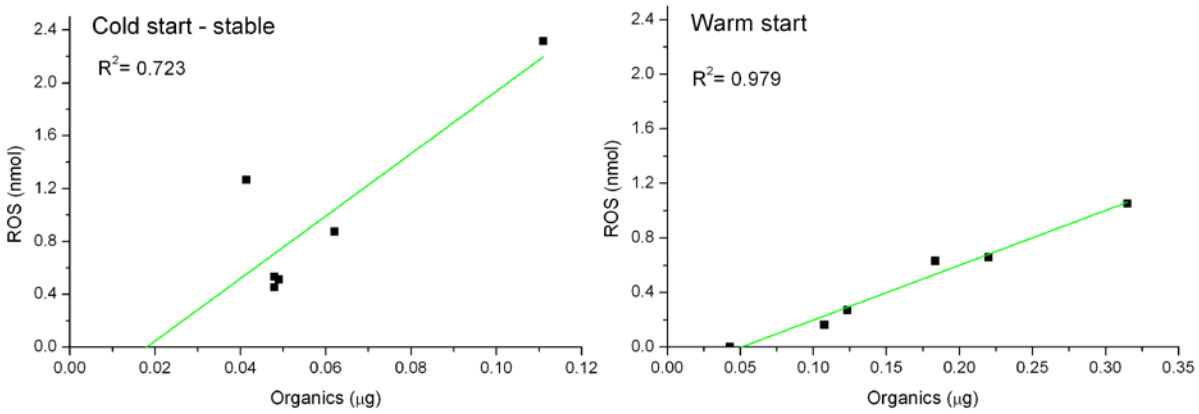

Figure **2** Correlation between the amount of ROS and the amount of organics for stable phase of cold-start (A), and warm-start (B) logwood burning.

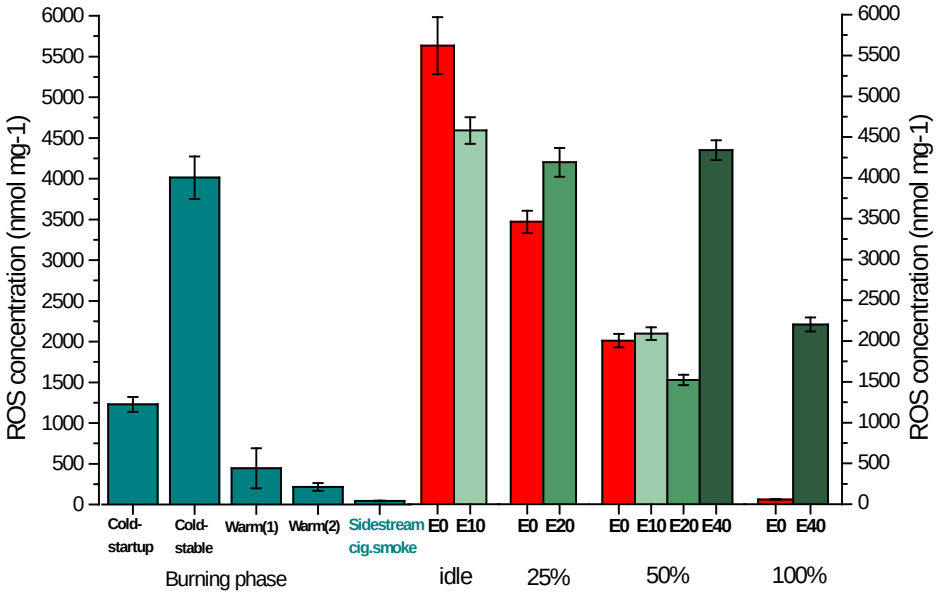

Figure **3** The amount of ROS for stable phase of cold-start (A), and warm-start (B) logwood burning, side stream tobacco smoke and different operating conditions for ethanol blended diesel