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Abstract

Reactive oxygen species (ROS) and free radicals play key roles in the chemical transformation and adverse health effects of aerosols. Their time-resolved speciation and quantification present significant challenges. This work presents a new approach for rapid sampling of radical species using a packed column coated with a profluorescent nitroxide scavenger, proxyl fluorescamine (PF), a probe selective to free radicals (R•). Quantification was performed by extraction and analysis using HPLC with fluorescence detection. The method was successfully applied for detection of R• in aging sidestream cigarette smoke as well as in secondary organic aerosols (SOA) generated from the ozonolysis of nicotine. For comparison, dichlorofluorescin (DCFH) was used as a reference probe for non-specific detection of a broad range of ROS in the same air samples. High R• and ROS concentrations (48 and 127 nmol mg^{-1} of equivalent H_2O_2, respectively) were measured in fresh tobacco smoke. These levels indicate that high concentrations of ROS (780 ppbv) and R• (295 ppbv) can be generated by one cigarette in a 20-m^3 room, confirming that tobacco smoke is an important source of reactive species indoors. Although aerosol aging led to significant reductions after 22 hours, both R• and ROS concentrations remained at non-negligible levels: 11 and 52 nmol mg^{-1} respectively. During nicotine ozonolysis, R• formed predominantly in association with particles whereas ROS was found primarily in the gas phase (14 and 47 nmol mg^{-1} respectively). Furthermore, in the presence of ozone PF was a stable probe while DCFH produced high blanks that could lead to over-estimation of ROS in ambient particles or SOA formed by ozonolysis. This case study shows that using PF on solid supports provides a good method to measure free radicals generated in cigarette smoke. The method can be used without modification for other combustion sources and ambient aerosols.

Keywords: Indoor environment, ozonolysis, thirdhand smoke, reactive oxygen species (ROS), radicals, aerosol mass spectrometry

Introduction

Epidemiological and toxicological studies have shown increases in human mortality and morbidity due to exposure to airborne fine particulate matter (PM) [1-4]. Although the mechanisms of PM-related health effects are still incompletely understood, recent evidence has shown that significant adverse health effects may originate from oxidative stress initiated by the formation of reactive oxygen species (ROS) within cells [5-9]. ROS may be present either on the ambient particles themselves [10-13] or the particles may contain chemical species such as semiquinones [7, 14] that are capable of generating ROS when they deposit in the lung. As a result, measurement of particle-associated ROS is the focus of considerable attention in ongoing efforts to understand the mechanisms of the health effects associated with particles.

The term ROS refers to molecules such as hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH), as well as free radicals, *e.g.*hydroxyl (HO•), peroxyl (HOO•, ROO•), superoxide (O_2^{-}•). Precursors of ROS that include carbon-centered (C•) and nitrogen-centered (N•) radicals can also be considered as ROS. Free radical species have characteristics that make them difficult to capture and detect, namely their very high reactivity, short lifetimes and low concentrations. Because of its high sensitivity and simplicity in data collection [15] an excellent approach to quantify ROS in aerosols is to measure the fluorescence induced when probe molecules encounter ROS. . Most studies have focused on the use of non-specific probes that respond to a variety of ROS. These include 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) [16-20], Dithiothreitol (DTT) [11-12, 21], dihydrorhodamine 123 (DHR-123) [22], and dihydrorhodamine 6G (DHR 6G) [23]. Despite their high sensitivity, the lack of specificity [24] and poor stability of these probes over time (e.g., autooxidation of DCFH-DA [25-26]), limit their

suitability for elucidating health effects of particles. Probes designed to detect specific groups of ROS with high selectivity are desirable, since each ROS has unique physiological activity. Recently some attractive fluorescent probes that exhibit higher selectivity toward specific ROS have been investigated, such as p-hydroxyphenylacetic acid (POHPAA) to detect hydroperoxides in urban aerosols [27], and proxyl nitroxides [28] to measure free radicals (R•) [29-32]. The latter type of probe consists of a fluorophore connected to a nitroxide group. Their interaction inhibits fluorescence until the radical scavenger nitroxide encounters carbon-, nitrogen-, and/or sulfur-, centered radicals. The reaction products produce stable fluorescent alkoxyamines at nearly diffusion controlled rates (10^7–10^9 M^{-1} s^{-1}) [28, 33].

Flicker and Green [34] and Bartalis et al. [31, 35] used 3-amino-2,2,5,5,-tetramethyl-1-pyrrolidinyloxy (3AP) to trap carbon-centered radicals (C•) in mainstream cigarette smoke. However, an additional step of derivatization was required after trapping to produce highly fluorescent products. More recently, Miljevic et al. [32] synthesized a new profluorescent nitroxide (BPEA-nit) that can be used in impingers for direct trapping and detecting of particle-derived ROS in cigarette smoke. The goal of this study is development of a simpler and more stable method for free radicals (R•) in combustion and secondary aerosols. The approach involved coating the profluorescent nitroxide proxyl fluorescamine (PF) [36-37] on solid supports, with subsequent HPLC-fluorescence analysis. For comparison, DCFH was used as a general indicator of ROS. Two model aerosols were tested in this study because of their ubiquity and known generation of ROS and radicals: Secondary organic aerosol (SOA) from ozonolysis reactions and diluted sidestream cigarette smoke [38], also known as secondhand smoke (SHS). SHS is a very common combustion-generated aerosol and a major source of ROS in indoor air. Furthermore, ROS and free radical mechanisms play central roles in tobacco smoke carcinogenesis, oxidative stress and cardiovascular diseases [9, 39-45].

Experimental

Materials and chemicals

Fluorescent probes: Proxyl fluorescamine (PF), 5-(2-carboxyphenyl)-5-hydroxy-1-[(2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl]-3-phenyl-2-pyrrolin-4-on potassium salt, is a radical scavenger whose nitroxide moiety quenches the fluorescence of its nearby fluorphore group with its delocalized unpaired electron. Once PF traps a radical R•, the fluorescence is restored by the conversion of the nitroxide to the corresponding spin-paired alkoxylamine (R'-N-OR) (Figure 1-A) [37, 46-47]. PF and DCFH: 2',7'-dichlorodihydrofluorescein diacetate, were purchased from invitrogen™ (Carlsbad, CA) and used without purification. H_2O_2 solution (≥30%, TraceSELECT®) and Horeseradish peroxidase type II were purchased from Sigma-Aldrich (St Louis, MO). SKC® Midget Impingers (25 mL) with fritted nozzles were used for sampling with DCFH. The solvents (acetonitrile, water, dimethyl sulfoxide) were of the highest purity possible (HPLC grade). The glass beads used for trap loading were 3 mm diameter (Fisher Scientific). were conditioned by washing sequentially with hexane, acetone and water then dried and stored in the dark at room temperature.

Preparation of probes for reactive species

Proxyl fluorescamine: Five grams of clean glass beads were loaded into a 100-mL round bottom flask. A 5-mL aliquot of PF (100 mg L^{-1}) in water was added to the beads. The contents of the flask were mixed well manually and then dried by gentle heating at 40 C in air. The coated beads were stored at -10 C prior to use, for up to 2 days. For sampling, coated glass

beads were loaded into stainless-steel tubes (Perkin Elmer, 15 cm length and 0.6 cm diameter) with a stainless steel mesh on the sampling inlet end. A small plug of glass wool on the exit held the beads in place. After sampling, the tube was flushed with 3 mL of acetonitrile and shaken gently for 5 min, before analysis using HPLC with UV/visible and fluorescence detection (details below).

Dichlorofluorescin (DCFH): 2',7'-dichlorodihydrofluorescin (DCFH) becomes oxidized to the fluorescent 2',7'-dichlorofluorescin (DCF) in the presence of a broad range of ROS (Figure 1-B). DCFH was generated from its diacetate (H_2DCFDA) by alkaline hydrolysis: (a) 0.0049 g of H_2DCFDA was dissolved in 1 mL methanol, (b) 500 μL of this 10 mM H_2DCFDA solution was added to 2 mL of 0.01 N NaOH and kept in the dark at room temperature for 30 min, and (c) 10 mL of a 25 mM phosphate buffer at pH 7.4 was added to the H_2DCFDA/methanol/NaOH solution. Horseradish peroxidase (HRP) was added to make the concentration 2.2 units HRP/mL to catalyze the reaction of DCFH and ROS. For ROS sampling, 300 μL of this solution was added to 15-mL aqueous solution in an impinger so that the final concentration of H_2DCFDA was 10 μM.

HPLC analysis of fluorescent probes

Samples were analyzed by high performance liquid chromatography (HPLC, Agilent 1200 series) equipped with a UV/visible and a fluorescence detector, on a C-18 column (4.6 x 150 mm). DCFH and its derivative (DCF) were eluted with a mobile phase 55/45 Methanol/H_2O (modified with 20 mM Na_2PO_4 pH: 6.8) at flow rate of 0.7 mL min^{-1}. Fluorescence of DCF was excited at λ_{exc} of 488 nm and quantified by λ_{em} at 530 nm. PF was eluted with a 65/35 acetonitrile/water mobile phase at a flow rate of 0.7 mL min^{-1}. PF was excited with λ_{exc} at 390 nm and measured with λ_{em} at 490 nm.

Generation of tobacco smoke and secondary aerosol samples

Secondhand smoke (SHS): SHS was generated by smoldering one cigarette from a leading US brand in 5-L glass vessel. During the first few minutes after smoking started the smoke was pumped into 100-L Tedlar bags filled with zero air at about 70% capacity. To investigate the effect of aging, SHS aerosol was left inside the bag in the dark at room temperature for 22h and in absence of any other reactive species. ROS samples were collected and characterization of particles was carried out at 0.5h, 4h and 22h after generation of SHS.

Secondary organic aerosols (SOA): The reaction of nicotine with ozone was used as a model system to generate SOA, as described previously [48]. 5μL of liquid nicotine (Sigma Aldrich, purity 99%) was injected into each of two 40-L Tedlar bags (A and B), filled with 30-L dry air (RH ~ 0 %). Two L of ozone (1.6 ppmv) was generated by UV irradiation (UVP Inc.) of pure air, and introduced into Bag B, corresponding to an initial concentration of 100 ppbv inside the bag (in the absence of reactive losses). Both bags were aged at room temperature in the dark for 30 min before ROS sampling. The ozone concentration in the supply air was measured using a calibrated UV Photometric Ozone monitor, model API 400 (Advanced Pollution Instrumentation, Inc.)

Chemical characterization of SHS and SOA samples

For particle characterization, Tedlar bags containing SHS and SOA were characterized without further dilution using a scanning mobility particle sizer (SMPS, model 3936, TSI, Shoreview, MN) and a custom built aerosol mass spectrometer (AMS, Aerodyne) that vaporizes the aerosol on a heated deposition plate, followed by photoionization with tunable VUV as

detailed elsewhere [48-49]. For the experiments reported here, the aerosol was ionized by 10.5 eV radiation produced by the Chemical Dynamics Beamline at the LBNL Advanced Light Source.

ROS and R• samples were collected immediately before AMS analysis, for a duration of 20 min using (a) a 25-mL impinger containing 15 mL of DCFH-HRP reagents, at a sampling flow rate of 0.5 L min^{-1} and (b) two tubes of PF-coated glass beads, connected in series, at a flow rate of 0.1 L min^{-1}.

Results and discussion

Stability of fluorescent probes

The stability of fluorescent probes is important to ensure the reliability of the method for practical application in the field. Thus, the stabilities of DCFH and PF standards were examined. When incubated at room temperature and in the dark for 24h, the DCFH working solution of 10 μM showed auto-oxidation, as illustrated by an increase of fluorescence background signal by a factor of ≈ 3. To minimize this effect, stock solutions of DCFH were stored in dark at -10 C, and working solutions were prepared daily before sampling. On the other hand, glass beads coated with PF and stored in stainless-steel tubes at room temperature were found to be stable for at least 24 h without generation of any artifact signal. The results show that PF solid supports do not require daily preparation and hence can be very practical for use in field measurements of free radicals.

Linearity, Precision, and Detection Limit

As ROS include a variety of reactive compounds, it is difficult to establish a calibration curve for each chemical. Thus, calibration of the system was performed with standard H_2O_2 solutions with concentrations ranging from 0.1 to 2 μM, prepared by serial dilutions of a 30% stock solution of H_2O_2, with MilliQ water serving as a blank [20, 50]. For DCFH, about 0.1 mL of H_2O_2 standard solution was mixed with 3 mL of the probe dissolved in 50 mM potassium phosphate buffer at pH 7.4, along with 2.2 units/mL HRP. The procedure followed in this study was quite similar to that of Hung et al. [20]. For calibration of PF we used the procedure developed by Blough et al. [51-52] based on the reaction between the hydroxyl radicals and dimethyl sulfoxide (DMSO) to form methyl radicals ($\bullet CH_3$), which then react with PF to yield the fluorescent hydroxylamine derivative of fluorescamine. Hydroxyl radicals were generated in solution through Fenton's reaction by mixing 0.1 mL of standard concentrations of H_2O_2 with 10 μM iron(II) sulfate heptahydrate ($Fe(SO_4).7H_2O$ and PF (10 mg L^{-1}) in a final volume of 3 mL of 5% DMSO in H_2O. The standard solutions and blanks were then incubated at room temperature for 30 min before HPLC analysis. As shown in Figure 2, both methods were linear with high correlation coefficients (R^2 > 0.97) over the range of studied H_2O_2 concentrations. The *equivalent H_2O_2 concentration* is an indicator of the reactivity of the ROS or R• in a sample that had fluorescence intensity identical to that of an H_2O_2 solution of the calculated concentration:

$$\chi_{air} = \frac{C \times V_e}{V_s} \quad (1)$$

$$\chi_{mg} = \frac{C \times V_e}{W} \times 10^3 \quad (2)$$

Where χ_{air} is the total concentration of ROS or R• in particulate and gas phases, expressed in nmol H_2O_2 m^{-3} of air; χ_{mg} is the concentration of ROS or R• in the particle phase, expressed in nmol H_2O_2 mg^{-1} of particles; C is the concentration of ROS in DCFH-HRP or R• in PF solutions, expressed in units of μM; V_e is volume of DCFH-HRP or PF solution, expressed in liters; V_s is sample volume of SHS or THS, expressed in m^3; w is the particle mass, determined by integrating the SMPS size distribution in the range 20-700 nm, expressed in milligrams, assuming a density of 1.1 g cm^{-3} for SHS particles [53]. This normalization is important to correct for the loss of SHS particles via deposition during aging.

Bubbling tobacco smoke through the DCFH impinger is not 100% efficient and values shown in Figure 4 are not corrected for the size dependent collection efficiency [54]. However, since the mass median diameter for SHS is between 200-300 nm, [32, 55], it is expected that the majority of particle mass gets trapped in the impinger. In addition, since both fresh and aging SHS exhibited similar size distributions, it is reasonable to assume that the comparison is valid. Results from the PF sampling method were corrected for breakthrough which was less than 20% in all experiments.

The precision of the HPLC analyses was determined from the reproducibility of four HPLC injections of DCFH and PF standards under the same experimental conditions over a period of 2 days. The maximum RSD was 9.6% for DCFH and 6.2% for PF. The method detection limit, defined as three times the standard deviation of the blank samples, was estimated to be 7 nM and 16 nM of equivalent H_2O_2 for DCFH and PF, respectively. These detection limits are comparable to those observed in prior studies using DCFH (5.8 nM) [16] and nitroxide probe (3 nM) [51].

ROS and R• in fresh and aging secondhand smoke

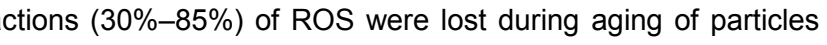
Due to the dynamic nature of tobacco smoke, it is essential to investigate the temporal dependence of ROS and R• during the time between emission of SHS and inhalation (minutes to hours) in the indoor environment. To evaluate the effect of SHS aging on ROS and R• levels, samples collected at three aging periods (1, 4 and 22h) were treated with PF and DCFH. Simultaneously, chemical composition and size distribution of SHS particles (1 and 4h) were monitored in real-time as shown in Figure 3. Figure 4 presents the time evolution of H_2O_2 equivalent concentrations for SHS during aging in the Tedlar bag. ROS and R• concentrations in fresh SHS were 127± 19 and 48 ± 7 nmol mg^{-1} equivalent H_2O_2 as measured by DCFH and PF, respectively. This difference is due to the fact that PF method measures primarily R•, whereas the DCFH method is a non-specific measure of the oxidative capacity of SHS, also including contributions of molecular species such as H_2O_2 and organic peroxides. Assuming an average SMPS integrated emission factor for SHS particulate of 5 mg per cigarette [56], the H_2O_2 equivalent concentration of ROS in fresh SHS would be 635 nmol/cigarette whereas (R•) would be around 240 nmol / cigarette. Table 1 compares the results from this study to previously reported concentrations of ROS in cigarette smoke. Although a quantitative comparison is limited due to differences in instrumentation, cigarettes brands, and sampling protocols, it is possible to draw some general conclusions:

A) Using the DCFH-HRP probe, our results show that one cigarette could produce a total ROS (gas and particles) concentration right after smoking of 32 nmol m^{-3} (1.1 mg m^{-3} or 0.78 ppmv of equivalent H_2O_2), assuming a typical 20 m^3 room size. These levels are in general agreement with those of See et al. [38] who measured ROS particle-bound concentration of about 10 nmol/m^3/cigarette for 8 m^3 room mechanically ventilated at 0.5 air change per hour. In

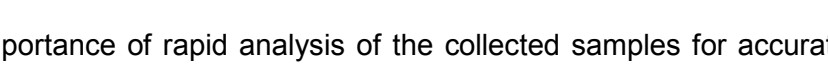
addition, reported background levels of ROS indoors are 2-4 nmol m^{-3} [38] and typical ROS values in ambient air of urban sites in the U.S. are 6 - 12 nmol m^{-3} (average 8.3 ± 2.2) [19]. Thus, it is clear that cigarette smoke represents a significant source of human exposure to airborne ROS.

B) R• levels in SHS measured by PF (48± nmol mg^{-1} ; 240± nmol/cigarette) are the same order of magnitude of those recently reported by Miljevic et al. (50 nmol mg^{-1}), using a nitroxide probe (BPEAnit) [32]. In a 20-m^3 room, emissions from one cigarette would increase R• by 295 ppbv H_2O_2 equivalents. Flicker at Green [34] reported 54 nmol of free radicals per cigarette (equivalent to 11 nmol mg^{-1}) when passing mainstream smoke (MSS) directly through a column filled with the nitroxide trap, 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (3AP). This suggests that SHS is an equally important or even greater source of R• than MSS. Lower levels of R• in MSS might be due to the cigarette filter and/or a lower fire cone temperature compared to SHS emitted between puffs or smoldering, leading to “less reactive” particles in MSS. While it is difficult from this study to infer the nature of the R•, they may include semiquinone (QH•) [57], acyl (R-C•=O) and alkylaminocarbonyl radicals (R-N-C•=O) [58].

During aging both ROS and R• concentrations in SHS decreased significantly. After 22 h, 40% of the initial ROS measured by DCFH remained in the sample, indicating that a significant fraction was lost during sample storage in the Tedlar bag. This finding also implies that ROS loss rate in a room will be at least the same order of magnitude. The R•/ROS concentration ratio almost halved, from 38% to 21%, during the first 22 hours of aging. Free radicals (R•) decreased quickly at the rate of ≈ 8 nmol mg^{-1} h^{-1} in the first hours due to possible reactions with other radicals and molecular species or scavenging by oxygen molecular O_2 [34]:



$$R\bullet + O_2 \rightarrow RO_2\bullet \quad (3)$$



$$RO_2\bullet + R'O_2\bullet \rightarrow ROOR' + O_2 \quad (4)$$

This R• loss behavior is consistent with previous studies where, compared to fresh particles, significant fractions (30%–85%) of ROS were lost during aging of particles from ozonolysis of linalool over 24 h and aging of ambient aerosols over115 h [20]. The loss of reactivity over time shows the importance of rapid analysis of the collected samples for accurately measuring the activity of reactive species. On the other hand, the presence of residual fractions of ROS (40%) and R• (23%) after 22h aging suggests (i) the existence of long-lived species such as organic peroxides, and stabilized radical species [59], and (ii) continuous formation of ROS and R• within smoke plumes via dynamic processes involving reactions of species such as NO, NO_2, O_2, and incompletely oxidized organic compounds [60-64]. Because radicals are formed within the aging smoke, they can be biologically important when smoke is inhaled, and can play an important role in the formation of hazardous chemicals in aged tobacco smoke, recently dubbed "thirdhand smoke" (THS) [65-68].

In an effort to identify the chemical changes induced by ROS and aging, we examined the mass spectra and size distribution of fresh and aged SHS particles. Compared to fresh (0.5 h) SHS, aged (4 h) SHS exhibits a particle size distribution that is slightly narrower, with a much lower concentration (380 $\mu g.m^{-3}$ vs 890 $\mu g.m^{-3}$ at 0.5 h). These differences are expected due to the higher rate of deposition loss [56] loss of large (>300 nm) particles by deposition to the walls of the Tedlar bag. The chemical composition showed remarkable similarities between fresh and aged SHS. In both cases, the most intense signals are assigned to nicotine at *m*/*z* 162 and its pyrroldine fragment (m/z 84). A list of the observed mass signals assigned to cigarette smoke constituents was previously reported [48]. The only noticeable change is the loss of nicotine signal by a factor of 5, likely due to its volatilization from the aerosol phase into

the gas phase with subsequent sorption onto the surfaces of the Tedlar bag [69-70]. All other m/z signals are similar and there is no evidence of new organic compounds formed in the particle phase. In addition, no correlation was observed between the composition of SHS particles and ROS content.

ROS in Secondary Organic Aerosols

Our previous work showed that nicotine, a major constituent of tobacco smoke, can be depleted substantially by its reaction with ambient ozone over timescales of hours to weeks that are competitive with desorption and re-emission into the gas phase [48, 71-73]. This reaction leads to the formation of stable gas phase products as well as secondary organic aerosol (SOA) in the ultrafine range (< 100nm) [48, 72], constituting part of thirdhand tobacco smoke (THS). This new term refers to residual tobacco smoke pollutants that persists after smoking ceases and could be reemitted from surfaces into the gas phase or react with oxidants or other compounds present in the environment to form secondary contaminants [66].

In this study we investigated the formation of ROS during nicotine ozonolysis, which could also contribute to health risks from exposure to THS. Figure 5 shows the concentrations of reactive species produced (THS) in the nicotine reaction with ozone, using DCFH and PF probes. Two blank samples were generated to account for the independent contributions of nicotine and ozone to the measured signal. The initial concentrations of nicotine and ozone in the blank samples and ROS sampling procedure were identical to the THS samples (nicotine + ozone). In addition, by placing a Teflon filter between the THS bag and the inlet of the DCFH impinger and PF solid trap, the aerosol from the reaction was isolated from the gas phase THS so that the contribution of the gas phase to ROS production could be measured. It is worth

mentioning that some losses of gas phase species to the filter could occur in this approach and/or ROS could evaporate from the particles.

As expected, very little ROS signal was measured for pure nicotine, suggesting that it does not interfere with the measurement of ROS using DCFH (see Figure 5). On the other hand, ozone (100 ppbv) produced a significant background fluorescent signal when using DCFH, illustrating the reactivity of DCFH towards ozone and indicating that previous measurements of ROS in SOA produced by ozonolysis could have overestimated the ROS production due to SOA. Using PF, very little signal was observed due to either nicotine or ozone. This finding confirms the selectivity of PF for measuring R• concentrations and it highlights the suitability of PF for measuring free radicals in ambient air where high levels of ozone are often reported.

After ozone and nicotine reacted for 30 min, the total concentrations of fresh ROS increased by a factor of 5-8 compared to ROS background levels. The dominant fraction of ROS produced (76%) was associated with the gas phase of THS. In contrast, the majority of R• was particle-bound, which could be an indication of their low-volatility and/or a stabilization mechanism in the particle phase. These results could also indicate that R• may play a role in the formation of some of the higher molecular weight species – oligomers - observed in SOA [74-75].

On a per SOA mass basis and assuming a particle density of 1 g cm^{-3}, the level of particle-bound ROS measured by DCFH was around 180 nmole mg^{-1} of equivalent H_2O_2. This concentration was higher than that produced by fresh SHS (127 nmole mg^{-1}), indicating that SOA in THS could be an important source of ROS indoors. This high ROS activity in SOA could be associated with the ultrafine size range of SOA (i.e. large surface area in ultrafine particles).

Furthermore, the ROS levels produced were comparable to those previously reported by Chen et al. [76] for SOA generated by ozonolysis of limonene (140-160 nmoles mg^{-1}). Thus, potential health effects cannot be ignored given the observed elevated SOA and ROS intensities. In addition, since ozone reaction with nicotine takes place mainly on the pyrrolidine nitrogen [48], we could also expect the production of reactive nitrogen species (RNS) such as nitric oxide (NO•), •NO_2, as well as alkylaminocarbonyl radicals: R-NH-C•(O). These species can also play important roles in both physiological and pathological processes upon exposure to SOA [77].

Implications for exposure assessment

Determination of R• by trapping these species on solid supports coated with nitroxide radical scavengers offers significant advantages over conventional fluorescent probes for measurement of total ROS such as DCFH. These advantages include the simplicity and convenience of sampling onto solid supports, the high efficiency of trapping free-radicals in a solvent-free system and the stability of the probe and radical adducts. In addition, this approach can simplify analysis of R• separately in gas and particle phases in complex matrices such as SHS, THS and combustion sources.

This study provides further evidence that secondhand tobacco smoke is an important source of ROS and free radicals (R•). Exposure to reactive species and ultrafine particles in aging tobacco smoke could increase associated health risks to the smoker and passive smokers [78]. The results presented in this work may help to develop more accurate models to assess non-smokers' exposures to secondhand and thirdhand smoke indoors. Furthermore, the new methodology could also provide a sensitive means for rapid monitoring of free radicals levels in other aerosol systems (wood smoke, exhausts from vehicles, etc). When used in

parallel with DCFH or another non-specific ROS probe, the new method for (R•) has the potential to differentiate cellular damage due to (R•) from that due to total ROS in ambient air.

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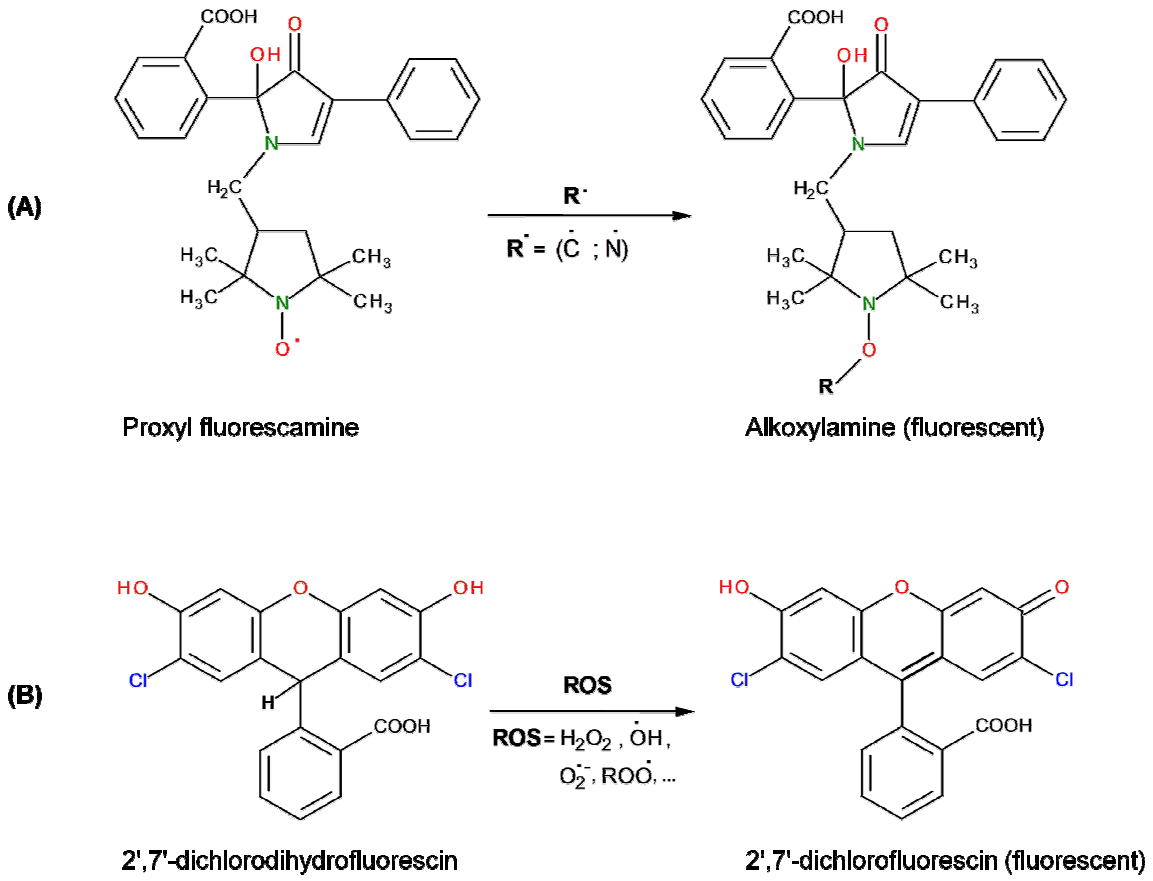

Figure 1. Fluorescent probes used in this study and their reactions mechanisms with ROS. (A) Proxyl fluorescamine, (B) Dichlorofluorescin.

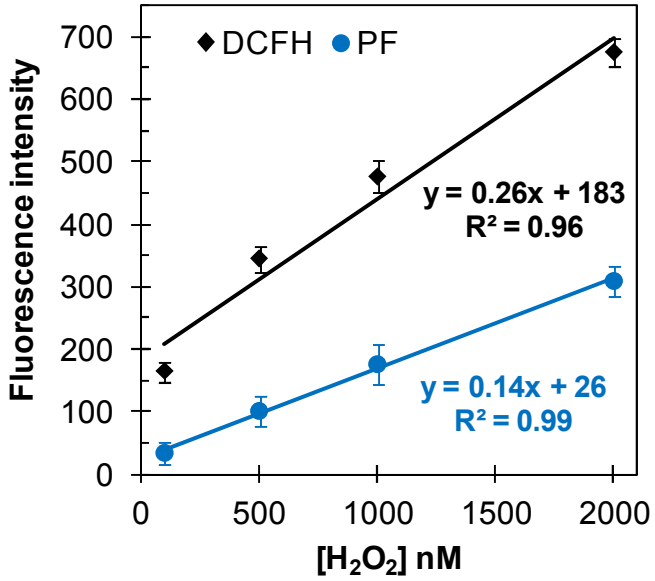

Figure 2. Calibration curves for Dichlorofluorescin (DCFH) and Proxyl-Fluorescamine (PF)

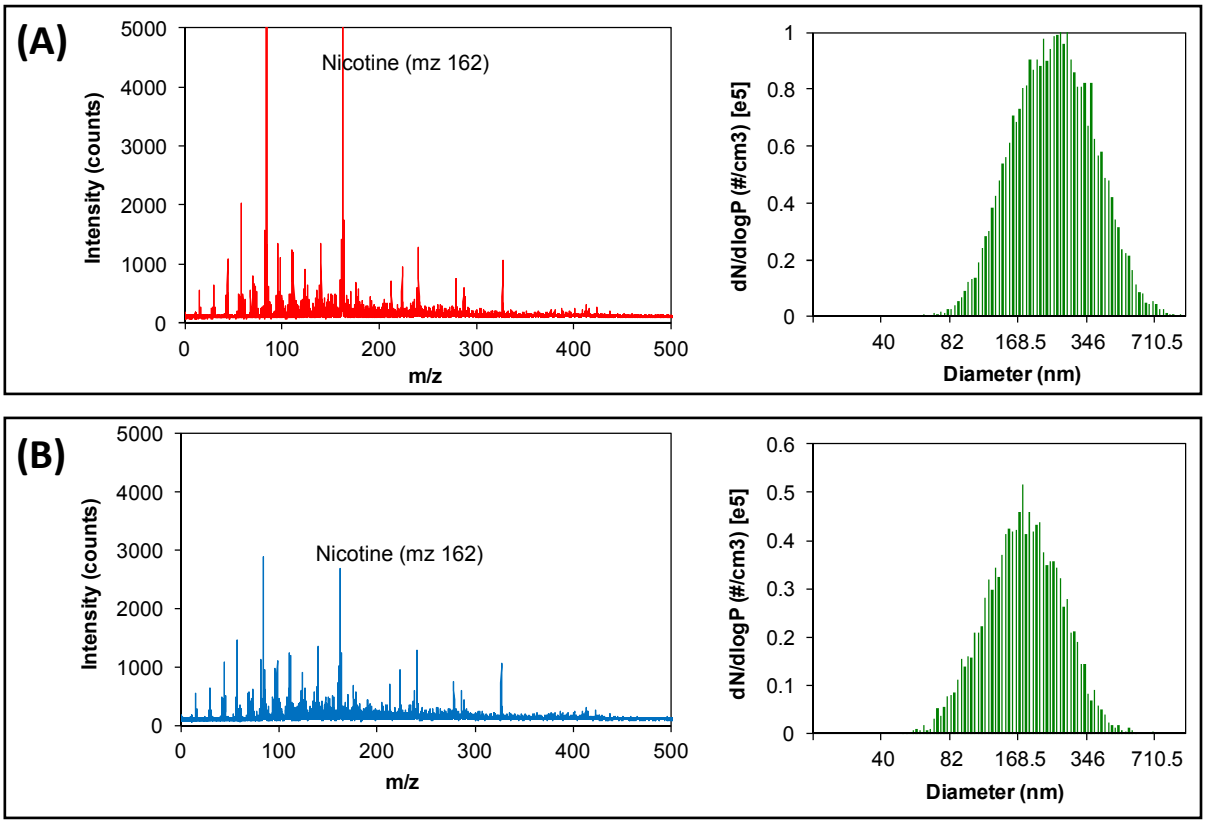

Figure 3. Mass spectra and size distribution of fresh 0.5 h (A) and 4-h aged (B) secondhand smoke.

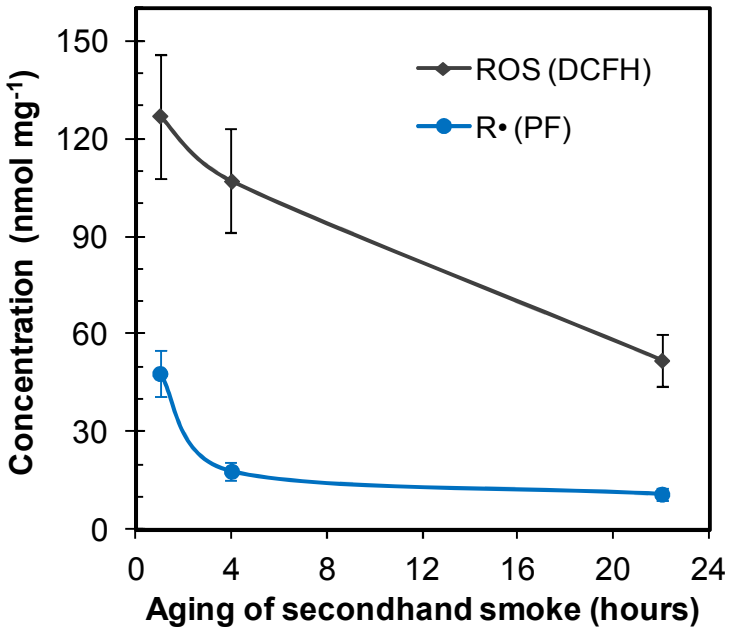

Figure 4. Temporal evolution of ROS and R• concentrations during aging of secondhand smoke

Table 1. Comparison of ROS and free radicals (R•) levels with previous studies

	Cigarette smoke [(a)]	Probe	Sampling approach	Concentration (nmol mg^{-1})	Ref.
ROS	*MSS*	DCFH-HRP	Impinger, 0.5 Lmin^{-1} for 1 min	3.68	[16]
		DCFH-DA	Collection on Quartz filters	10-11	[79]
	SHS	DCFH-DA	Collection on Quartz filters	3.1	[79]
		DCFH-HRP	Collection on Teflon filter	115	[38]
		DCFH-HRP	Impinger, 0.5 Lmin^{-1} for 20 min	127	This study
R•	*MSS*	3AP	Supported 3AP & derivatization	11[(b)]	[34]
		BPEAnit	Impinger, 1 L min^{-1} for 20 min	101	[32]
	SHS	BPEAnit	Impinger, 1 L min^{-1} for 20 min	50 ± 2	[32]
		PF	Solid support coated with PF	48	This study

[(a)] MSS: mainstream smoke; SHS: secondhand smoke; [(b)] concentration was converted from nmol/cig to nmol mg^{-1} assuming 5mg as total particulate mass emitted by cig

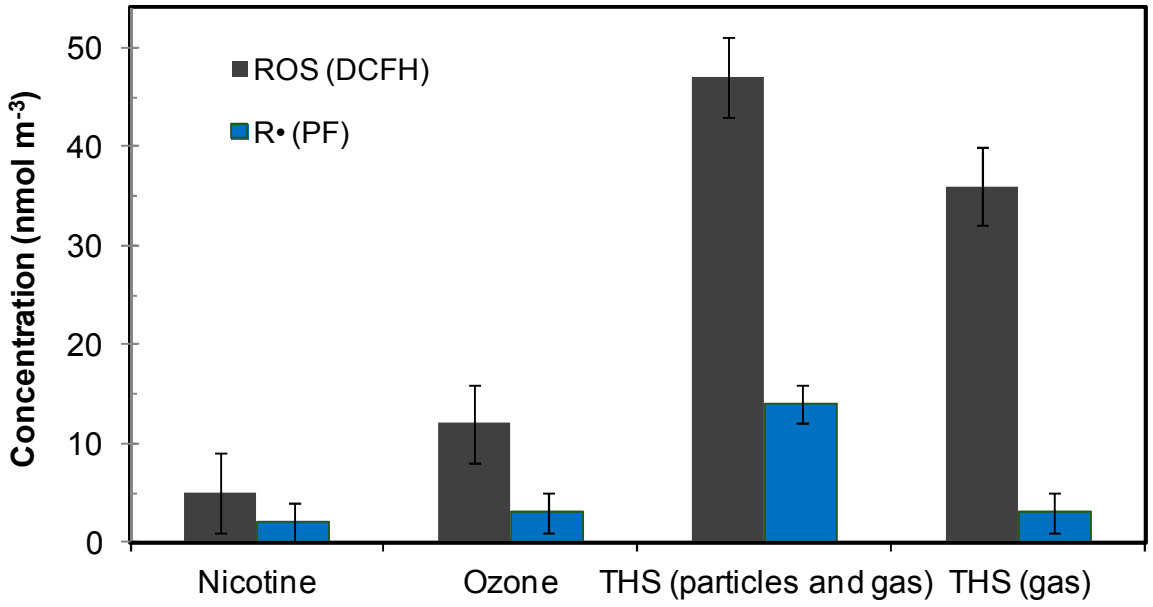

Figure 5. ROS and R• levels in secondary aerosol generated by nicotine ozonolysis

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