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Interference from carbonaceous particles in reactive oxygen species measurements: A case study with Printex 90 and BPEAnit

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Abstract

Reactive oxygen species (ROS) and oxidative potential (OP) are strongly associated with adverse health outcomes and have emerged as a more health-relevant metric for assessing the toxicity of air pollution than particle number or mass concentration. Acellular ROS and OP measurements rely on specific chemical probes that enable quantification through fluorescence or absorbance. During measurements using a real-time ROS monitor with 9,10-bis (phenylethynyl) anthracene-nitroxide (BPEAnit) as the probe, we observed a reduction in the fluorescent response when sampling particles containing high levels of black carbon, ostensibly indicating negative ROS values, although there is scientific evidence that black carbon can generate ROS. In this study, we investigated this interference from black carbon particles in BPEAnit solution in order to understand the underlying mechanisms and propose a method of correcting for the artefacts during ROS measurements.

We suspended Printex 90 particles, known to induce OP, at three different concentrations (1 μg mL^{-1}, 5 μg mL^{-1} and 25 μg mL^{-1}) in solutions containing either BPEAnit, its fluorescent and non-reactive methylated derivative BPEAnit-Me, or the solvent used in the probe solutions, dimethyl sulfoxide (DMSO), alone. The fluorescence of the suspensions was measured in the flow-through measurement cell from the real-time ROS monitor at different times after the addition of the particles. For suspension in BPEAnit and BPEAnit-Me, we found that fluorescence decreased with increasing particle concentrations, whereas no decrease was observed in solvent-only suspensions. The fluorescence response of BPEAnit with particles was consistently higher than for the same particle concentrations in BPEAnit-Me. Based on the results, we suggest that the main mechanism for the decrease in fluorescence is interactions between the particle surfaces and BPEAnit/BPEAnit-Me, and not light scattering or absorption. We attribute the difference in fluorescence between BPEAnit-Me and BPEAnit to ROS formation.

The observed interaction mechanisms are likely not specific to BPEAnit, and similar artefacts should be considered when assessing ROS or OP of carbonaceous, and possibly other insoluble particles, in both online and offline assays. For online ROS measurements with BPEAnit, we suggest regular use of BPEAnit-Me to detect and potentially correct for artefacts caused by insoluble particles.

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1 Introduction

Epidemiological studies have consistently linked exposure to airborne particles, mainly PM2.5, to adverse health effects(Cohen et al., 2017; Who, 2024). A widely suggested mechanism of particle-induced toxicity is through oxidative stress(Bates et al., 2019; Horie and Tabei, 2021; Leni et al., 2020). Particles can contain reactive oxygen species (ROS), and/or generate ROS in the body after inhalation. ROS is a group of chemicals, including •OH, O_2•-, RO•, ROO•, H_2O_2, etc. (Sies et al., 2022). Exposure to particles that contain or generate ROS can deplete the body's antioxidant defense, creating oxidative stress (Li et al., 2003). The ability of particles to deplete the antioxidants is called oxidative potential (OP)(Bates et al., 2019; Rao et al., 2020). Measurement of ROS and OP is considered a crucial tool for assessing particle toxicity, and is regarded as a potentially more biologically relevant metric than mass or number concentration(Molina et al., 2020; Zhang et al., 2022; Tassel et al., 2025).

In the last two decades, several acellular assays have been developed to measure ROS and OP (Bates et al., 2019). These assays use different chemical probes that are quantified by fluorescence or absorbance in solution. The assays include, but are not limited to, dithiothreitol (DTT), ascorbic acid (AA), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), glutathione (GSH) and 9,10-bis (phenylethynyl) anthracene-nitroxide (BPEAnit). Since the probes are sensitive to different types of ROS, they elicit different results, despite being exposed to the same particles (Dominutti et al., 2023; Jovanovic et al., 2019). ROS/OP assays can be implemented using two measurement approaches: offline and online methods. In offline analysis, the particles are collected on filters and later extracted for use in the assay. In online (real-time) measurement, particles are collected directly in a liquid, and quantification is achieved within minutes to half an hour (Carlino et al., 2023). The main advantages of the online methods are the ability to detect short-lived ROS (Elkaee et al., 2025; Liu and Schauer, 2025) and capability of tracking dynamic changes in time. Several studies have found that a significant part of ROS is not measured in offline methods, where the prolonged analysis time makes the short-lived species degrade before quantification (Fuller et al., 2014; Campbell et al., 2025; Brown et al., 2020).

One online ROS analyser is the Particle into nitroxide quencher (PINQ) (Brown et al., 2019b). It uses the chemical probe BPEAnit dissolved in dimethyl sulfoxide (DMSO) (Brown et al., 2019b). BPEAnit is a pro-fluorescent chemical that is sensitive to a broad range of ROS and is relatively stable to auto-oxidation (Stevanovic et al., 2012). It reacts rapidly with ROS due to a diffusion-limited mechanism, making it suitable for online analysis. Like other pro-fluorescent nitroxides, it has a free radical that quenches the fluorescence. The nitroxide reacts with ROS, thereby removing the quenching of fluorescence. The molecule becomes fluorescent as a stable derivative (primarily the methylated derivative BPEAnit-Me), allowing for ROS quantification. BPEAnit-Me is typically synthesized to generate a calibration curve for PINQ, which is then used to calculate the equivalent ROS concentration from the PINQ measurements(Brown et al., 2019b).

Several studies show that insoluble particles generate ROS and contribute to OP(Verma et al., 2012; Fang et al., 2017; Gao et al., 2020). Insoluble particles contribute to ROS generation via their reactive surfaces, involved in catalytic reactions or electron transfer. Despite the risk of underestimating results, many assays remove insoluble particles before analysis is made (Wragg et al., 2016; Utinger et al., 2023; Puthussery et al., 2018; Charrier and Anastasio, 2012). One reason for the exclusion is that the particles might interfere with the optical reading during quantification (e.g. due to light scattering or absorption). The issue of interference from insoluble

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particles is believed to cause problems in fluorescence and absorbance assays used for purposes other than ROS or OP measurements(Kroll et al., 2012; Doak et al., 2009; Monteiro-Riviere and Inman, 2006). The interference is often explained by optical absorbance or adsorption of chemicals on particle surfaces, and the need for control tests is highlighted.

One type of insoluble particle of high toxicological relevance is black carbon (BC), formed from incomplete combustion of carbon-containing materials, including fossil and biomass fuels. It is a ubiquitous component of atmospheric particulate matter and can also be found at elevated concentrations in indoor environments (e.g. from candle burning). BC has received increased attention due to its potential impact on human health. Adverse health effects that have been linked to black carbon exposure include cancer, cardiovascular dysfunctions and respiratory diseases. In all of these health outcomes, oxidative stress has been identified as one of the key mechanisms(Niranjan and Thakur, 2017; Liu and Chan, 2022). BC found in outdoor and indoor environments can have a complex composition with varying levels of organic coating and ab/adsorbed inorganic components. For laboratory investigations aiming to understand the processes and mechanisms of interaction between particles and the probe, it is advantageous to use industrially manufactured analogues, carbon black, produced under controlled conditions and with well-defined properties. One common example of carbon black is Printex 90, which we used in our investigations.

During online measurements with PINQ, we observed a decrease in fluorescent response for particles that contain BC. This observation was the basis for this study, in which we aim to understand the underlying mechanisms, their effects on measured ROS, and how to mitigate them. The decrease in measured fluorescence when BC is present is in line with suggestions from other studies with different assays(Boyles et al., 2022; Utinger et al., 2025). We hypothesize that the interference might be caused by scattering or absorption of the light, or by deactivation/quenching of the probe by adsorption to surfaces of carbonaceous particles, creating an underestimation of the measured ROS concentration. This study aims to explain the interference from black carbon particles in the BPEAnit assay, and suggests methods to correct for the observed decrease in response during online measurements with PINQ.

2 Method

To investigate the interaction mechanisms between carbonaceous particles and the BPEAnit probe during ROS measurements, we used the measurement cell from the online ROS analyzer PINQ (Brown et al., 2019b) for fluorescence measurements. All experiments were conducted using Printex 90, a well-defined, commercially available carbon black, which served as a proxy for soot particles. Experiments were conducted to investigate the optical effects, role of particles' concentration and temporal development of measured fluorescence.

To isolate the effect of particles on measured ROS, all measurements were performed both in the presence and absence of particles (Printex 90) in three types of solutions:

1. DMSO only (solvent used in the BPEAnit and BPEAnit-Me solutions) to check for possible optical interference with the light due to the presence of particles

2. BPEAnit (reactive probe) to check effects of different concentrations of Printex 90 and temporal development of ROS generation

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3. BPEAnit-Me (stable, fluorescent, non-reactive probe) to check possible optical interference, effects of different concentrations of Printex 90 and temporal development of fluorescence reduction

2.1 PINQ

The PINQ's cell (Brown et al., 2019a), used for fluorescence measurements, consists of a 4,5 mW laser (Collimated laser diode, Thorlabs) with a wavelength of 450 nm that illuminates liquid drawn through a glass tube enclosed in a stainless steel casing. The liquid is continuously drawn at a flow rate of 1 mL min^{-1} by a peristaltic pump (Masterflex, Ismatec). The inner diameter of the tubing and glass tube is 1 mm. The tube leading to the measurement cell is covered with aluminum foil to prevent light interference with the BPEAnit solution. Perpendicular to the laser is an optical fibre, which leads to a spectrophotometer for the detection of fluorescent light (Ocean HDX, Ocean Optics). Before the spectrophotometer, there is a high-pass filter that removes light below 460 nm, protecting the sensor from stray laser light. The illuminated liquid is discarded.

2.2 Probe solutions

DMSO (ACS grade, VWR Chemicals) were used for preparation of BPEAnit and BPEAnit-Me solutions. BPEAnit, synthesized by Jice (Guangdong) Instruments Co. Ltd (Zhuhai, China), was dissolved in DMSO at a concentration of 4 µM, corresponding to the concentration applied in PINQ measurements. The solution was wrapped in aluminum foil for light protection. To ensure a stable solution, it was stored in a laboratory cabinet at room temperature for 24 hours before use.

A stock solution of BPEAnit-Me, synthesized by Jice (Guangdong) Instruments Co. Ltd (Zhuhai, China), the fluorescent, chemically stable derivative of BPEAnit, was dissolved in DMSO at a concentration of 1µM. It was stored in a laboratory cabinet at room temperature. From the stock solution, 130 nM BPEAnit-Me was prepared for experiments. This concentration was chosen to match the background fluorescence level associated with BPEAnit at 4 µM (as described in detail in data analysis).

2.3 Printex 90

A stock suspension of Printex 90 (Degussa, DE) at 1 mg mL^{-1} in DMSO, was prepared before experiments. To remove aggregates and obtain a homogeneous particle suspension, the stock suspension was sonicated in an ultrasonic bath (Elmasonic Easy 100H, Elma) for 30 minutes. Care was taken to keep the bath temperature below 35°C.

2.4 Conducted experiments

2.4.1. Optical effects

To detect if the presence of particles causes reduction of light intensity due to scattering and/or absorption, the fluorescence was measured from suspension of particles in DMSO and compared to particle-free DMSO. Two Falcon 50 mL tubes were filled with DMSO. A small amount

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of the stock suspension with Printex 90 was placed in one of the Falcon tubes, resulting in a final particle concentration of 25 µg mL^{-1}. The tubes were vortexed (Vortex-Genie 2, Scientific Industries) for ten seconds before starting measurements. From the Falcon tubes, the liquid was drawn by the peristaltic pump through the measurement cell.

Additionally, to check optical effects, a suspension of Printex 90 (5 µg mL^{-1}) in 130 nM BPEAnit-Me was used. The suspension was filtered through a syringe filter (Cole-Parmer) with 0.22 µm pore size. The fluorescence of the filtered suspension was compared to that of the unfiltered suspension.

2.4.2. Role of concentration

To investigate the effect of particle concentration, the experiments were conducted at different Printex 90 concentrations in BPEAnit and BPEAnit-Me solutions.

The stock suspension (1 mg mL^{-1} in DMSO) was used to create suspensions in BPEAnit and BPEAnit-Me solutions, at a particle concentration of 25 µg mL^{-1} in Falcon tubes. The tubes were vortexed for ten seconds to create a homogeneous suspension. Directly after vortexing, an aliquot of each 25 µg mL^{-1} suspension was diluted to 5 µg mL^{-1} in a second tube. The procedure was repeated to acquire a third suspension, with a particle concentration of 1 µg mL^{-1}. The suspension concentrations have been chosen to 1) match concentrations at which our laboratory investigations of candle smoke (manuscript in preparation) were performed (i.e. 150 µg m^{-3} of BC, which is equivalent to 2.5 µg mL^{-1} in suspension), and 2) due to comparability to DCFH analysis, where the particle concentration during ROS measurements is normally set as a dilution series between 1 µg mL^{-1} and 100 µg mL^{-1} (Boyles et al., 2022). All samples were covered with aluminum foil to prevent the influence of light on the probe. The measurements were conducted within five minutes after addition of particles to the BPEAnit/BPEAnit-Me solutions. Before starting measurements in the measurement cell as described above, each sample was vortexed again for ten seconds to avoid particle settling. ROS was measured in each sample for a minimum of two minutes. All measurements were done in triplicate, with new particle stock solution and new probe solutions for each replicate.

2.4.3. Temporal development

To further evaluate whether particle–probe interactions influence the fluorescent response of BPEAnit, time-resolved measurements were performed on all samples described in the section above. To assess the temporal evolution of ROS generation and particle–probe interactions, fluorescence was measured on the same suspension at multiple time points, namely: 5 minutes (as described in the section above), 30 minutes and 1, 2, 3 and 24 hours. The suspensions were stored at room temperature. Before each measurement, the samples were vortexed for ten seconds to avoid particle settling. Note: BPEAnit is typically not used in different timescales, but its immediate reaction is used for detection of ROS in real-time measurements in PINQ. Here, these tests were of an exploratory nature to understand the mechanisms.

2.5 Data analysis

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BPEAnit and BPEAnit-Me have an emission wavelength of 483 nm. The secondary peak has a wavelength of 515 nm. During measurements, the spectrophotometer was set at a temporal resolution of 1 Hz and the entire spectrum was logged every 20 seconds. The spectrophotometer measured each solution for a minimum of two minutes. The fluorescence response was calculated by integrating over the primary fluorescent peak, at an interval of 480-486 nm. After removing the first 30 seconds of each measurement period, due to a transient response, an average of the response was made.

Even in the absence of ROS, the BPEAnit solution exhibits fluorescence, since not all fluorescence is fully quenched. The concentration of BPEAnit-Me (130 nM) was chosen to match the baseline fluorescence in BPEAnit (4 µM), enabling comparisons of the changes in fluorescence between BPEAnit and BPEAnit-Me in the presence of particles. The ratio between the chosen concentrations is similar to the ratio between the quantum yields for BPEAnit and BPEAnit-Me (Fairfull-Smith and Bottle, 2008). The fluorescent response of the suspensions was normalized to particle-free solutions (baseline fluorescence) of BPEAnit and BPEAnit-Me, respectively.

3 Results

In Fig. 1, results from previous experiments are shown, namely fluorescence counts at BPEAnit emission peaks when measuring no particles, laboratory-generated secondary organic aerosols (SOA, formed from reactions between alpha-pinene and ozone), and candle smoke. Aerosols were generated in an aerosol chamber under controlled conditions and measured with PINQ. SOA and candle smoke mass concentrations were comparable. The fluorescence from SOA is higher than the control, where no particles are sampled. This increase is attributed to particle-bound ROS reacting with the probe. In contrast, when sampling candle smoke, the fluorescence decreased compared to the control. This decrease was unexpected, since soot has been shown to generate ROS(Liu and Chan, 2022; Li et al., 2024).

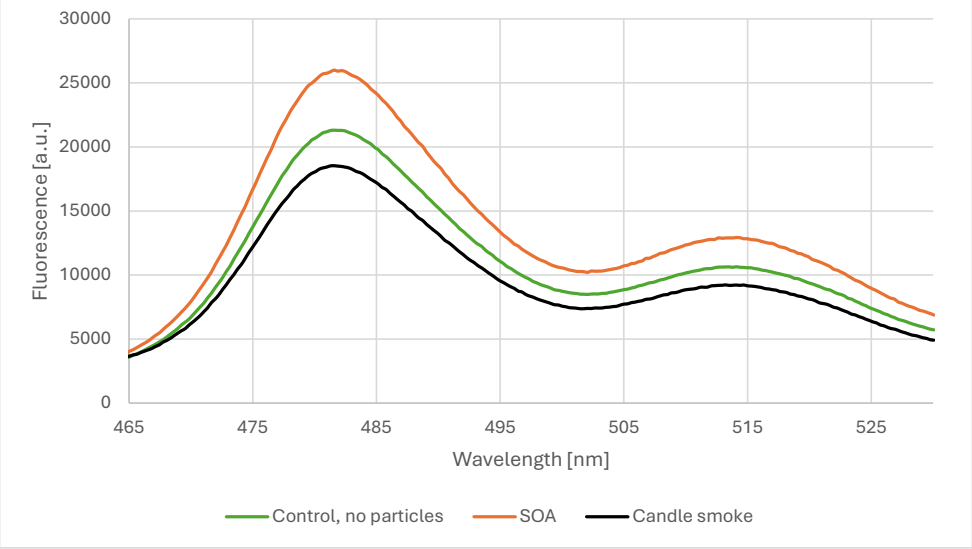

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Figure 1. Fluorescence emissions in arbitrary units of 4 µM BPEAnit during sampling of clean air (control, no particles), SOA and candle smoke. Data from PINQ measurements in laboratory conditions. Primary (483 nm) and secondary (515 nm) fluorescent peaks of BPEAnit are visible.

3.1. Optical effects

No difference in fluorescence response was observed when the fluorescence spectrum from DMSO with and without Printex 90 (25 µg mL^{-1}) was compared (see Fig. A1 in appendix). Additionally, the difference between filtered and unfiltered suspension of Printex 90 in BPEAnit-Me in average relative fluorescence was about 3 % (see Table A2 in appendix). Namely, 55 % for the unfiltered suspension and 58 % for the filtered one. The small difference of 3 % indicates that light being scattered or absorbed by the particles can be considered negligible, i.e. not being the main factor of fluorescence loss.

3.2. Role of concentration

The fluorescence response from suspensions of different particle concentrations, in BPEAnit (pro-fluorescent) and BPEAnit-Me (fluorescent and non-reactive), is shown in Fig. 2.

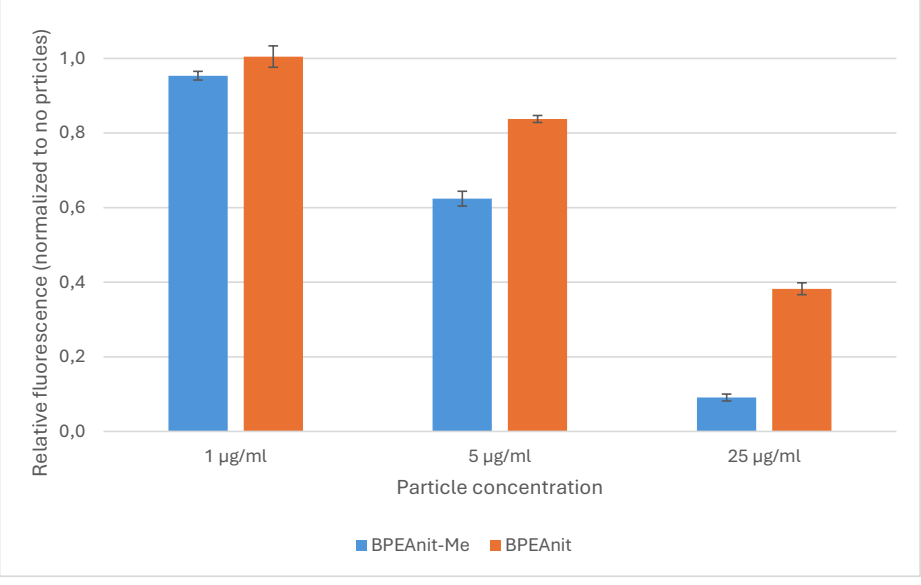

Figure 2. The relative fluorescent response (normalized to no particles) for suspensions with Printex 90 at different concentrations in BPEAnit-Me and BPEAnit solutions, measured within 5 minutes after adding the particles to the solutions. Result shown is average ±SD.

The response decreases for all experiments, except for 1 µg mL^{-1} P90 in BPEAnit, where no change was seen. Increasing the particle concentration gives a greater decrease in fluorescence. The relative response is higher for BPEAnit than BPEAnit-Me.

3.3. Temporal development

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In Fig. 3, the relative fluorescence response for all concentrations at multiple time points after adding particles to the probe solutions is shown.

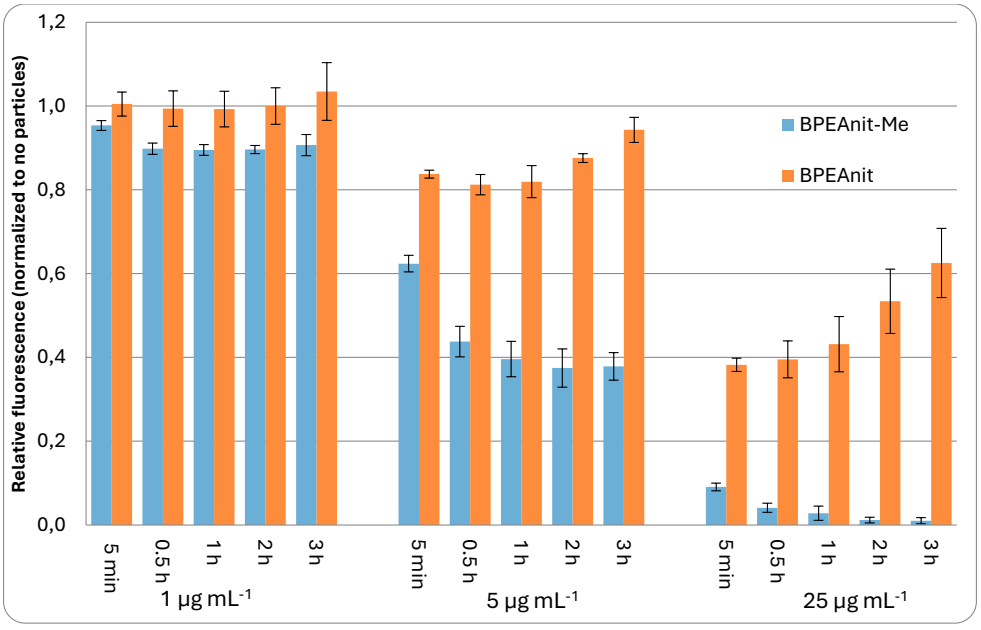

Figure 3. Fluorescence from suspensions with Printex 90 at 1, 5, and 25 µg mL^{-1} in solutions with BPEAnit or BPEAnit-Me measured at different time points 5 min, 0.5, 1, 2 and 3h. Fluorescence values are normalized to the no-particle condition and shown as mean ± SD.

For BPEAnit-Me, most of the decrease occurs within 30 minutes; thereafter, the response plateaus. For BPEAnit, there is an increase in response over time, but it does not reach a plateau. After 3 hours, the response is still increasing. Measurements were also done after 24 hours, presented in appendix (Fig. A3), showing further increase for BPEAnit and very little change for BPEAnit-Me, compared to 3 hours.

In Fig. 4, the fluorescent response from a suspension of 5 µg mL^{-1}, in both BPEAnit-Me and BPEAnit, measured within 5 minutes, is shown. The difference in response between BPEAnit-Me (63 %) and BPEAnit (84 %), which we interpret as ROS-generated response, is marked.

To calculate the ROS contribution, F(ROS), the following equation was applied:

$$F(ROS) = \frac{(F(BPEAnit) - F(BPEAnitMe))}{F(BPEAnitMe)} \quad (1)$$

Where F(BPEAnit) and F(BPEAnit-Me) are the relative fluorescence (normalized to no particles) in BPEAnit and BPEAnit-Me, respectively. The following assumptions were made: a) the particle-probe interaction is identical for both BPEAnit-Me and BPEAnit due to the chemical similarities; b) the concentration difference in BPEAnit-Me and BPEAnit is compensated by the difference in

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quantum yield of the two probes and the use of relative values, i.e. normalized to no particles (baseline fluorescence). Details are explained in the Discussion section.

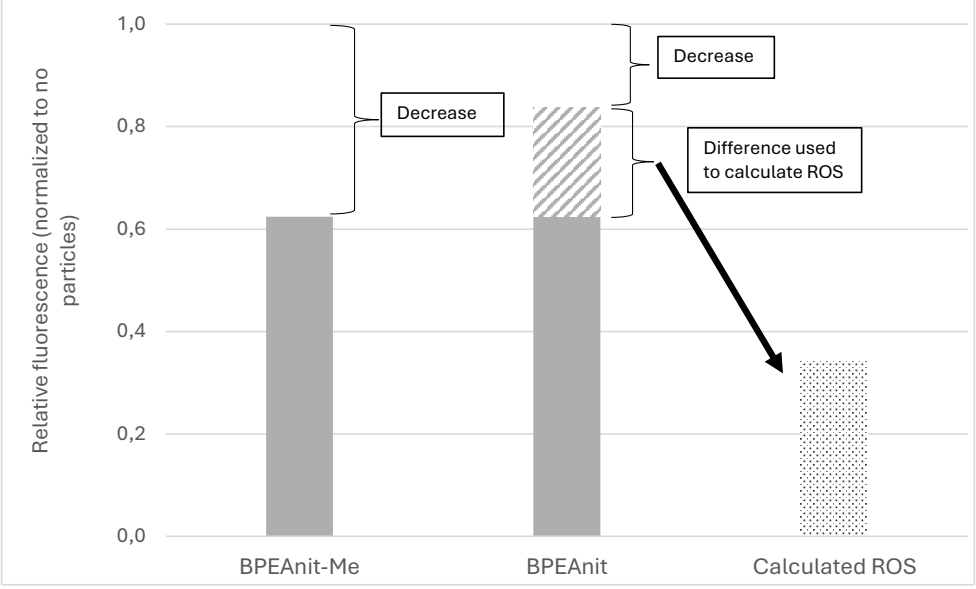

Figure 4. The relative fluorescence for 5 µg mL^{-1} Printex 90, measured within 5 minutes after mixing the particles in the two probe solutions (first two bars, equal to F(BPEAnit) and F(BPEAnit-Me) in Equation 1) and calculated ROS (equal to F(ROS) in Equation 1). Calculated ROS represents the ROS generated response after correcting for loss of fluorescence after particle-probe interaction. "Decrease" visualizes the reduction in fluorescence relative to no particles.

In Fig. 5, the calculated (i.e. corrected) fluorescence (details in Discussion), interpreted as generated ROS, is shown and grouped by measurement time. Values in Fig. 5 are based on the difference between the response from BPEAnit and BPEAnit-Me in Fig. 3. It can be seen that higher concentration and longer reaction time increase the ROS response.

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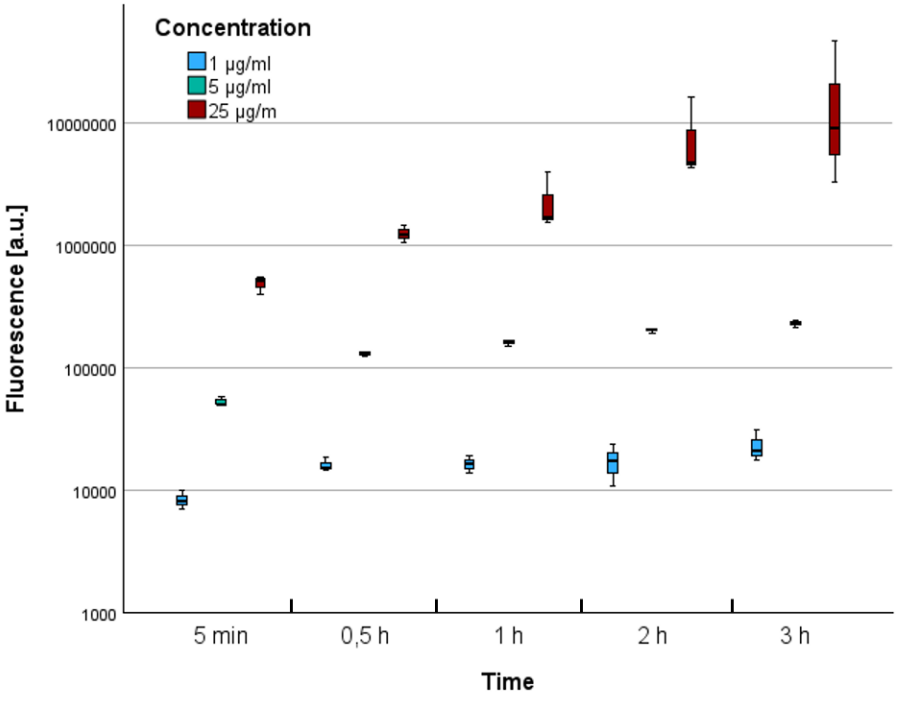

300 **Figure 5.** Calculated ROS (based on Equation 1) for P90 in BPEAnit at different concentrations, measured at different times after particle addition.

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4 Discussion

The experiments performed to investigate optical effects (presented in Fig. A1 and Table A2) show that light scattering and light absorption are not the main reasons for the observed artefact. Additionally, if the light absorption and scattering played a major role, the response for Printex 90 in BPEAnit-Me at three different concentrations in Fig. 3 should have remained unchanged over time, whereas a decrease is observed. Together, these results support our hypothesis that particle interaction with the probe is responsible for the reduction in fluorescence. One suggested mechanism of fluorescence reduction is ad/absorption of the probe onto particle surfaces, leading to inactivation of the fluorophore(Valderrama et al., 2008; Matte et al., 2011).

Considering the chemical similarities between BPEAnit and BPEAnit-Me (only one bond changed), we assumed identical ad/absorption onto particle surfaces. Both BPEAnit and BPEAnit-Me will be affected by the reduction of fluorescence from particle-probe interactions, but only BPEAnit will elicit an increase in fluorescence due to reactions with ROS. Based on previous findings from other ROS assays, Printex 90 is known to generate ROS in e.g. DCFH and DTT assays (Sauvain et al., 2013; Liu and Chan, 2022; Boyles et al., 2022; Malmborg et al., 2025). In line with this, BPEAnit consistently exhibited smaller relative decrease than BPEAnit-Me in our study (Fig. 2), which is attributed to ROS formation induced by Printex 90. Therefore, the difference in fluorescence between BPEAnit and BPEAnit-Me is the ROS-dependent probe activation. We hypothesized two competing mechanisms influencing the measured ROS signal: a decrease due to particle-probe interactions and an increase due to ROS generation. Both mechanisms exhibit their own kinetics and differ depending on time scales. As shown in Fig. 3, the decrease in BPEAnit-Me fluorescence occurs already after 5 min (which is of relevance for PINQ measurements) and decreases further with increasing concentration of particles. However, the process continues to take place, and an even larger decrease in BPEAnit-Me is observed within one hour, whereas the fluorescence increase attributed to ROS generation continues for 3 hours.

The measurements presented in Fig. 2 were performed within five minutes after adding Printex 90 to the probe solutions. This is comparable to PINQ-measurements, in which analysis is made within one minute after collection and mixing of the aerosol in the BPEAnit solution. Even at the lowest investigated particle concentration, 1 μg mL^{-1}, the fluorescent decrease in BPEAnit-Me is evident within five minutes (5 % compared to the fluorescence without particles). As a comparison, the particle concentration in the probe liquid would be 1 μg mL^{-1} during PINQ measurements of an aerosol concentration of 63 μg m^{-3}, assuming 100 % collection efficiency. The corresponding aerosol concentration for the 5 μg mL^{-1} suspension would be 315 μg m^{-3}. The particle mass concentration of candle smoke during PINQ measurements in laboratory conditions (shown in Fig. 1) was around 150 μg m^{-3} and elicited a decrease in fluorescent response of 13 % compared to the control without particles. This indicates that the particle concentrations chosen in this study are comparable and highly relevant for the interpretation of the laboratory PINQ measurements.

One of the major advantages of online ROS monitors, including PINQ, is the ability to capture short-lived ROS. However, in Fig. 3, a drawback of that approach is observed. The response increases if the particles and the probe react for a longer time. For exposure assessment, it is important to determine the full effects of particles deposited in the respiratory tract, including both short-lived ROS and ROS generated over time by insoluble particles, which are not captured by online methods. Therefore, the use of both online and offline instruments would be

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recommended during ROS and OP measurements. The results presented here suggest that BPEAnit could be explored as a probe for OP measurements.

To correct for observed artefacts, i.e. to calculate ROS, we suggest using Eq. (1). The difference between the fluorescence of BPEAnit and BPEAnit-Me is attributed to ROS generation, since we assume identical particle-probe interaction in the suspensions. Upon reaction with ROS, BPEAnit forms a fluorescent product. However, this product is also likely to adsorb to particle surfaces, resulting in loss of fluorescence. This is accounted for with the denominator in the equation, assuming that the newly formed product is suppressed in the same ratio as the BPEAnit-Me solution under identical experimental conditions. As illustrated in Fig. 4 (for 5 µg mL^{-1} Printex 90), approximately 63 % of the BPEAnit-Me fluorescence remains after mixing with Printex 90 particles. Consequently, the ROS-attributed fluorescence, calculated as the difference between the BPEAnit and BPEAnit-Me responses, is divided by 0.63 to correct for fluorescence loss due to particle interactions.

We hypothesize that there are two possible conditions that can introduce errors when correcting the artefacts using Eq. (1). **Condition A:** *Effect of depletion of BPEAnit-Me at high particle concentration:* In Fig. 3, it can be seen that the relative fluorescent response for BPEAnit-Me at a particle concentration of 25 µg mL^{-1} is only a few percent. This means that BPEAnit-Me is almost completely depleted. To avoid such depletion, probe concentration should be increased at high particle loads. One outcome of BPEAnit-Me depletion is that the calculated ROS, shown in Fig. 5, is greatly increased, since the denominator in Eq. (1) approaches zero. **Condition B:** *Effects of decreased adsorption of newly formed fluorescent product at low particle concentration (due to difference in adsorption rate for BPEAnit and BPEAnit)*: Equation 1 is based on the assumption that the particle-probe interaction is identical for both BPEAnit-Me and BPEAnit. At very low particle concentrations, a higher BPEAnit concentration (compared to BPEAnit-Me) may result in a greater fraction of possible adsorption sites on the particles being occupied. This would mean that the newly formed fluorescent product (after reaction with ROS), would adsorb to the particles at a lower rate than that of BPEAnit-Me, and lead to higher measured fluorescence (Gehlen, 2020). This would lead to an overestimation of the calculated ROS. Please note that specific limits/threshold values are not yet known and would require detailed investigation. An alternative approach to artefact corrections is to assume that there is no adsorption of the newly formed BPEAnit-Me after ROS reaction. Given this assumption, only the nominator of the equation could be used; however, it would lead to an underestimation of ROS concentration. In the appendix (Fig. A4), the fluorescent ROS response is shown for this assumption.

When measuring ROS on particles with elevated BC concentrations (e.g. laboratory emission studies, combustion sources, extreme pollution events such as wildfires, etc.), care should be taken to account for the negative effect on fluorescence from carbonaceous particles. It is not fully understood to what degree the effects of low BC concentrations (at ambient concentrations within EU air quality limits) affect ROS measurements with PINQ. One study (Stevanovic et al., 2019) using BPEAnit in ambient measurements found non-linear responses at BC levels above 2 µg m^{-3}. Moreover, it is not yet known how other insoluble non-carbon black particles affect the probe; it requires careful assessment through targeted studies. Until it is fully understood, as a precautionary principle, we propose a method that includes alternating between BPEAnit and BPEAnit-Me during PINQ measurements. The frequency of the alternation should depend on the sampled particle concentration and the stability of the aerosol properties. During stable conditions, BPEAnit-Me should be run for a quarter of the measurement time. The equation above is then used to calculate the particles' ROS contribution. If the particle concentration or aerosol

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properties vary continuously during the measurement, the time with BPEAnit-Me should be increased so that BPEAnit and BPEAnit-Me are used for equal amounts of time. This is to ensure that comparisons between the probes are made at similar particle concentrations, or to acquire sufficient data to allow for extrapolation of particle interference at different concentrations.

This study has focused on BPEAnit and online PINQ measurements. However, the effect of BC on ROS and OP measurement methods is likely not limited to this specific probe. It is known that high concentrations of nanoparticles, including Printex 90, decrease the response in the DCFH assay (Boyles et al., 2022; Kessler et al., 2021). To avoid issues in the offline DCFH assay, the procedure is to use only particle concentrations where no negative effect is visible, which is not possible when DCFH is used in instruments that run in real-time measurements. Our results show that particle-probe interaction occurs at lower concentrations as well, meaning that data points at the lowest concentrations taken for the ROS or OP assessment in offline methods could also be affected by a reduction in fluorescence. Similar interference has been observed in different *in vitro* toxicity assays (Kroll et al., 2012). These studies highlight the importance of appropriate controls to identify and correct for interference caused by insoluble particles. Until now, such controls have not been incorporated in measurements with BPEAnit. Other online ROS monitoring instruments could also be affected by the described particle effect (Utinger et al., 2025). Even instruments that remove insoluble particles before the fluorescent measurement could exhibit decreased fluorescence if particle removal occurs after probe addition, since removing particles from the probe solution does not restore the fluorescence signal (Table A2).

Limitations of this study include the limited number of particle concentrations of the suspensions. The tested Printex 90 concentrations (1, 5 and 25 µg mL^{-1}) cover a relevant concentration range, although testing additional concentrations would allow determination of a threshold below of which BC does not cause artefacts in ROS measurements. Non-linear effects were observed at atmospheric concentrations during field studies with the BPEAnit probe (Stevanovic et al., 2019), suggesting that even low BC concentrations cause artefacts. Another limitation is that the probe concentrations were kept constant (130 nM and 4 µM) throughout the study. Varied probe concentrations would enable further investigation of the mechanisms of the particle-probe interaction. Since probe depletion was apparent at 25 µg mL^{-1}, it would have been relevant to examine how the fluorescence reduction would change at higher probe concentrations. It would also be of interest to further study the role of probe concentration on adsorption rate at low particle concentrations. Even though our results clearly demonstrate the artefacts for carbonaceous particles, since only one type of carbonaceous particle (Printex 90) with specific and known characteristics was used, we cannot generalize the artefacts to any insoluble particles. Studying different types of BC and other types of insoluble particles would enable a better understanding of the role of e.g. surface area and surface chemistry in probe deactivation.

5 Conclusion

This study aimed to explain the effect of insoluble particles containing black carbon when measuring ROS with the BPEAnit probe. We found that the presence of carbonaceous particles, even at low concentrations (in BPEAnit-Me at 1 µg mL^{-1}, corresponding to atmospheric concentrations of 63 µg m^{-3}), causes a decrease in fluorescence response, compared to conditions without particles. Based on performed experiments, the results show that this decrease is primarily caused by inactivation of the probe due to ad/absorption to the particles. It

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was shown that the effects of light scattering and absorption are negligible. The results highlight the importance of robust control tests when measuring particles with black carbon. By using a stable derivative of BPEAnit, we suggest an approach to correct for the carbonaceous particle-induced fluorescent decrease for online and offline ROS measurements with BPEAnit. However, this effect might not be exclusive to BPEAnit and should be considered when assessing ROS from insoluble carbonaceous particles, in both online and offline assays. This study is limited to a single type of carbon black (Printex 90). Future research should include a broader range of particle types to determine whether the observed effects are specific to black carbons or applicable to other insoluble particles and to assess the role of particle surface area and surface chemistry. Forthcoming measurements using PINQ should incorporate and evaluate the suggested correction procedure.

This study has successfully described the interference of carbon black particles on BPEAnit during ROS measurements, and proposed a mechanism relevant to other ROS measurement methods. The understanding of artefacts in ROS and OP measurements is urgently needed since the new European regulation of air quality recommends measurements of OP at supersites. It is of high importance to advance beyond PM as a metric for particle-induced health effects.

Data availability

All data used in the manuscript can be made available upon request.

Author contribution

JE performed the analysis and wrote the original manuscript. Study conceptualized by JE and AW, with support from VM and ACE. Funding acquisition by AW. BM, HW and ZR provided scientific support. All co-authors contributed to the final manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Appendix A

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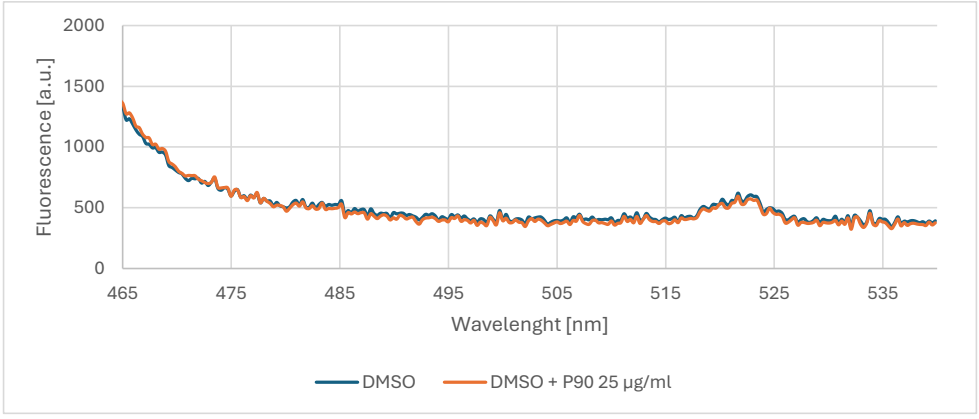

Figure A1. The spectrum of the fluorescence from DMSO in arbitrary units, with and without Printex 90 at a concentration of 25 µg mL^{-1}. Measurements were taken 30 minutes after the addition of particles.

Table A2. Fluorescence response for BPEAnit-Me suspension with a Printex 90 concentration of 5 µg mL^{-1}, measured before addition of the particles, after particle addition, and after filtration of the particles through a syringe filter with 0,22 µm pore size. The result is averaged from triplicate.

	No particles	Unfiltered	Filtered
Fluorescence (a.u.)	114278	62504 ± 3183	66181 ± 3956
Relative decrease		0,55	0,58

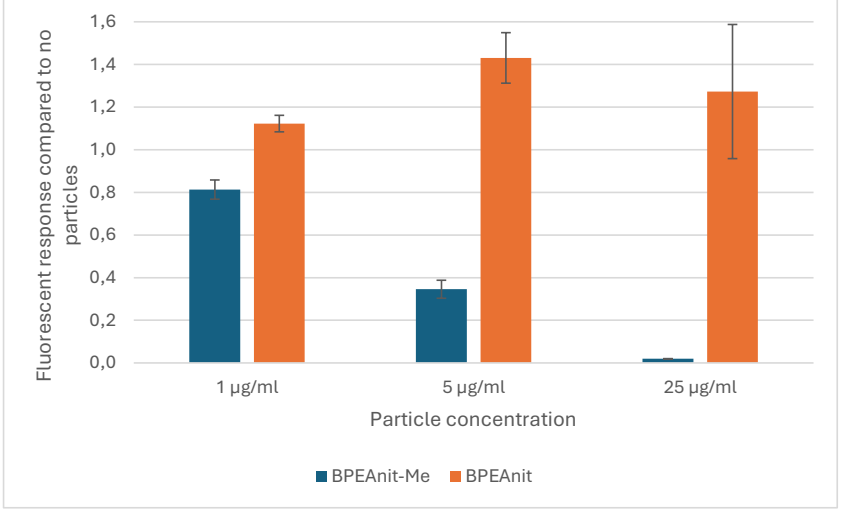

Figure A3. Fluorescence from suspensions with Printex 90 at 1, 5, and 25 µg mL^{-1} in solutions with BPEAnit or BPEAnit-Me, measured 24 hours after addition of particles. Fluorescence values are normalized to the no-particle condition and shown as mean ± SD.

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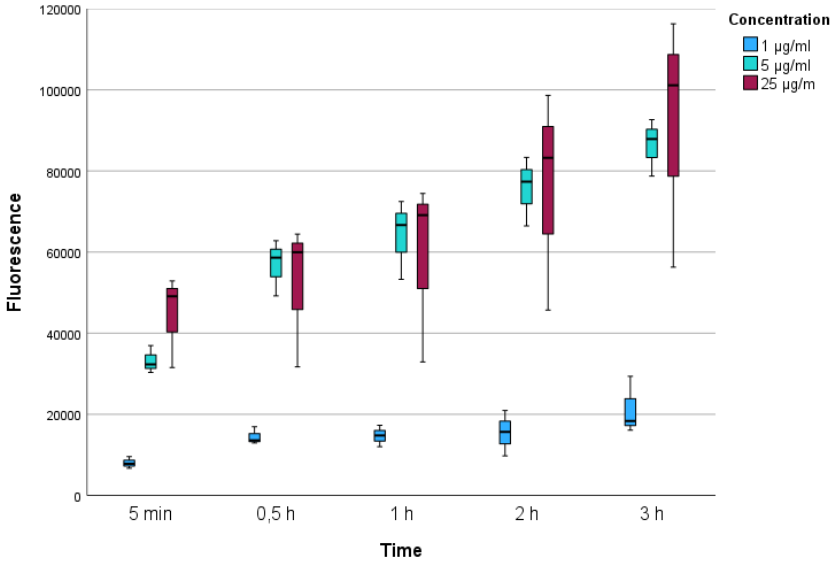

Figure A4. An alternate approach to estimate the ROS contribution (based on the differences between BPEAnit and BPEAnit-Me in Fig. 3) for P90 in BPEAnit at different concentrations, measured at different times after particle addition.

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